Jihočeská univerzita v Českých Budějovicích
Výzkumný ústav rybářský a hydrobiologický

Sperm Motility and Behavior in Models of Teleostean and Chondrostean Fish

Sayyed Mohammad Hadi Alavi

Vodňany 2009

University of South Bohemia České Budějovice
Research Institute of Fish Culture and Hydrobiology
Vodňany, Czech Republic

Sperm motility and behavior in models of teleostean and chondrostean fish

Sayyed Mohammad Hadi Alavi

Sperm motility and behavior in models of teleostean and chondrostean fish

Sayyed Mohammad Hadi Alavi

Rector of University of South Bohemia České Budějovice (Prof. PhDr. Václav Bůžek, PhD.) and director of USB RIFCH (Prof. Dipl.-Ing. Otomar Linhart, DSc.) will be award PhD. in fisheries (3rd of September 2009, at 2 p.m. in USB RIFCH) to the author of the thesis. From that time the author of the thesis can use the title PhD.

Supervisor:
Prof. Dipl.-Ing. Otomar Linhart, DSc.
University of South Bohemia České Budějovice (USB)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II
389 25 Vodňany
Czech Republic

Head of Laboratory of Reproductive Physiology in Fish:
Prof. Dipl.-Ing. Otomar Linhart, DSc.

Director of USB RIFCH:
Prof. Dipl.-Ing. Otomar Linhart, DSc.

Board of doctorate study defence with referees:
Prof. Dipl.-Ing. Vlastimil Baruš, DSc. – head of the board
Prof. MVDr. Zdeňka Svobodová, DSc. – board member
Prof. Dipl.-Ing. Petr Ráb, DSc. – board member
Assoc. Prof. RNDr. Zdeněk Adámek, PhD. – board member
Assoc. Prof. MVDr. Stanislav Navrátil, PhD. – board member
Ing. Miroslav Prokeš, Ph.D., ÚBO AV ČR Brno
Assoc. Prof. RNDr. Jana Pěknicová, PhD. – referee
Dr. Jacky Cosson, Ph.D., CNRS, France. – referee

Date, hours and place of PhD. defence:
3th of September 2009, at 11 a.m., USB RIFCH, Vodňany

Name: Sayyed Mohammad Hadi Alavi

Title of thesis: Sperm motility and behavior in models of teleostean and chondrostean fish

PhD. thesis, USB RIFCH, Vodňany, 2009, 107 pages, with the summary in English and Czech.

ISBN:

DECLARATION

I declare that this thesis hereby submitted by me, Sayyed Mohammad Hadi Alavi, to the University of South Bohemia in České Budějovice for the degree of Doctor of Philosophy in Zootechnique – Fishery has not been submitted by me for a degree at another university and that this is my own original work.

Contents

Chapter 1

An introduction to biology of sperm in fish

1- Introduction

In the fish farm, consideration of broodfish management is a prerequisite for successful artificial breeding and must include evaluation of methods for larval rearing and the cultivation of marketable size fish, artificially. Fish farms are usually stocked by broodfish that have been artificially reared or captured from natural bodies of water. In both wild stock and cultivated broodfish, there are many factors that act against the production of high numbers of good quality gametes. Successful management of broodstock remains of immense importance for ensuring the consistent production of valuable offspring to meet market demand. To date, a large number of studies have been focused upon the management of female broodfish. However, it is equally vital to manage the male broodfish, especially in species prone to the following deleterious conditions: low potentiality for milt production (suc as silurids) (Linhart et al., 2004), high risk of sperm contamination during stripping (such as carp, tench, silurids and marine fish) (Linhart et al., 2003b; Dreanno et al., 1998; Perchec-Popard et al., 1998) or reduced period of sperm viability (Suquet et al., 1994; Alavi and Cosson, 2006). Th management of male broodstock is highly species-specific and depends upon gonadal morphology, the physiology of sperm production, and the quality and quantity of sperm released. All of these parameters are heavily influenced by environmental factors which affect milt quality in terms of both physiology and biochemistry. Consequently, this can lead to the need to select a specific type of technique which to manipulate production (Billard et al., 1995a,b; Bromage and Roberts, 1995).

2- Morphology of testes and functions

Morphological structure of the reproductive accessory organs in male fish varies among different branches of the phylogeny tree, mainly according to reproductive behavior and ecology, thus confirming significant inter- or intra- specific variation (Grier, 1981; Nagahama, 1983; Parenti and Grier, 2004). In most fish, the male reproductive system consists of the testes, testicular gland, seminal vesicles, testicular main duct and spermatic duct (Billard et al., 1982; Nagahama, 1983; Patzner and Lahnsteiner, 1999). The testes are divided into “Lobular type” and “Tubular type” according to differentiation of the germinal tissue (Billard et al., 1982). Few years ago, Parenti and Grier (2004) classified testicular structure into “unrestricted spermatogonial testis” and “restricted spermatogonial testis”. Testes are containing both germ and somatic cells (Leydig and Sertoli cells). The testicular gland is located at the ventral side of the testis and its physiological function is similar to that of the spermatic ducts including differentiation of spermatids (similar to the Sertoli cells in the testes), nutrition of spermatozoa, secretion of mucin and phagocytosis and lipid storage. The seminal vesicles (specific glandular structures, Nagahama, 1983; Patzner and Lahnsteiner, 1999), functions are secretion of steroids, and storage of ripe spermatozoa. The testicular main duct is very important in nutrition of spermatozoa, storage of spermatozoa, synthesis of steroids, secretion of proteins and enzymes and formation of seminal fluid. The main roles of spermatic duct, where maturation of spermatozoa to gain potentiality for motility occurs, are including regulation of seminal plasma composition through secretion of ions, lipids, proteins, enzymes and steroids. Resorption and lysis of necrotic spermatozoa is additional physiological function of spermatic duct.

3- Endocrine regulation of spermatogenesis and spermiation in fish

Similar to higher vertebrates, reproductive activity in fish is regulated by the endocrine system consisting of hypothalamus-pituitary-gonad axis (see reviews by Billard et al., (1982), Nagahama, (1994), Yaron, (1995) Schulz and Miura (2002), Coward et al., (2002), Vizziano et al., (2008), Watanabe and Onitake (2008) and Mananos et al., (2009).

Stimulation of the release of gonadotropin releasing hormone (GnRH) and/or inhibition of gonadotropin release-inhibiting factor causes the pituitary to release gonadotropin (GTH). There are two different GTH called GTH-I and GTH-II secreted from the proximal pars distalis in fish, which are structurally similar to tetrapod follicle stimulating (FSH) and luteinizing hormone (LH), respectively (Suzuki et al., 1988a,b). GTH-I regulates early gonadal development while GTH-II control spermiation (final maturation) through stimulation of gonadal steroidogenesis. Similar to other vertebrates, a two-receptor model for Gth-I and GTH-II action on gonadal steroidogenesis has been proposed (Nagahama, 1994; Yaron, 1995).

Spermatogenesis in teleost fish takes place within testicular cysts formed by Sertoli cells. Within each cyst germ cell development occurs synchronously (Grier, 1981; Billard et al., 1982; Nagahama, 1983). The process of spermatogenesis can be devided into three major phases: (1) the mitotic proliferation of the spermatogonial, (2) the mitotic devision of the spermatocytes and (3) the transformation of the haploid spermatids into flaggelated spermatozoa. GTH-I stimulates secretion of androgens in males (testosterone and 11-ketotestosterone). At late spermatogenesis stage, GTH-II induces secretion of progestin-like steroids called maturation-inducing steroids, which are prerequisite for sperm maturation.

Following environmental stumuli and/or hormonal induction of spermatogenesis, GTH-I stimulates the Leydig cells for secreting 11-KT which in turns activates the Sertoli cells to produce activin B. Then Activin B acts on spermatogonial to induce mitosis leading to the formation of spermtocytes and subsequently spermiogenesis. Insulin-like growth factor-I is also necessary for the continuation of the process by stimulating DNA synthesis in spermatogonial (Loir and Le Gac, 1994; Le Gac et al., 1996; Nader et al., 1999). Miura and Miura (2003) mentioned that activin B only induces proliferation of spermatogonial, but not meiosis and further spermatogenesis. Recent study by Miura et al., (2006) clarified that a progestin, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), is an essential factor for the initiation of meiosis in eel (*Angullia japonica*).

Spermatozoa within the testicular lobules are immotile, and may lack fertilizing capacity (Yaron, 1995). For acquiring capacity for the initiation of motility, maturation of spermatozoa occurs through the endocrine regulators, GTH-II mediated mainly by progestins. 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and 17α,20β-21-trihydroxy-4-pregnen-3-one are two major progestins involving in sperm maturation (Ueda et al., 1985; Miura et al., 1991, 1992; Rahman et al., 2000; Barcellos et al., 2002), in the amplification of milt production (Baynes and Scott, 1985) and in the acquisition of the potential motility of male gamete (Miura et al., 1992; Miura and Miura, 2003). In some teleosts, it has been suggested that progestin receptors exist in spermatozoa (Thomas et al., 1997). As a result, GTH-II stimulates the testis to induce production of DHP, which acts directly on spermatozoa and induces the activation of eSRS22/CA and finally this enzymatic activity causes an increase in the seminal plasma pH (Nagahama, 1994; Miura and Miura, 2003). Finally, intracellular mediators such as cAMP (Miura et al., 1992; Cosson et al., 1991) regulate acquisition

of potentiality of sperm for initiation of motility (Morisawa and Morisawa, 1986, 1988).

4- Sperm morphology and fine structure

Morphological studies on spermatozoon are considered one of the major subjects in spermatology such as diversity of spermatozoon morphology and ultrastructure of spermatozoon. Studies on sperm morphology can provide us with information about phylogenetic implications in fishes and reproductive ecology and physiology. It has been reviewed by Baccetti et al., (1984), Mattei, (1991), Jamieson, (1991) and Lahnsteiner and Patzner, (2008).

Fish spermatozoon can fall into two main categories: (a) *Aquasperm:* sperm discharge into aqueous environment and is always in species with external fertilization. (b) *Introsperm:* sperm is not freed into ambient water and is almost in species with internal fertilization.

Uniflagellate spermatozoon is a basic teleostean aquasperm and called "primitive spermatozoon". It is divided into type I and type II. spermatozoon Type I has rounded or ovoid nucleus (approximately 2 – 3 μm in diameter), no acrosome two centrioles (proximal and distal present distal to nucleus) each consisting of 9 triplet microtubules and a flagellum. The proximal centriole is often located at right angles to the distal centriole, which forms the basal body of the flagellum. The distal centriole gives rise into the cytoplasm and the membrane is drawn inwards to form a cytoplasmic canal. Then a rotation causes the flagellar axis to become perpendicular to the base of nucleus. Mitochondria are present around centrioles and the base of the flagellum. The plasma membrane of the flagellum in some species extends 1, 2 or 3 longitudinal flattened processes termed "fins". This structure may help sperm to swim faster in aquatic (freshwater or marine) environment. In type II spermatozoon, the flagellum remains parallel to the base of the nucleus and rotation does not exist.

5- Seminal plasma

Biological roles of seminal plasma have been recently defined as a component that play main role in supporting spermatozoa and physio-endocrinological functions after release of sperm from testis to sperm duct and subsequently after discharge of sperm into aquatic environment (Alavi and Cosson, 2005, 2006). Therefore, studies on seminal plasma parameters is a prerequisite factor (a) to understand the basic biochemical processes occurring during maturation of sperm in male reproductive accessory organ (vas efferent or deferent) (Marshall et al., 1989; Loir, 1990), spontaneous motility of sperm in sperm duct (Nagahama, 1994; Miura and Miura, 2003; Cosson, 2004), initiation of sperm motility after release to external environment (Cosson et al., 1999; Morisawa et al., 1999; Krasznai et al., 2000; Alavi and Cosson, 2006), (b) to evaluate the reproductive ability inter- or intra-specificity (Billard et al., 1995a; Alavi and Cosson, 2005, 2006) and (c) to optimize procedures of artificial fertilization in fish farms by improving of sperm handling such as stripping method (opening of abdomen, catheterization, hand-stripping), fertilization techniques (wet or dry), and methods of short-term storage and long-term storage of fish semen (Billard, 1992; Billard et al., 1995a,b; Linhart et al., 2004).

Seminal plasma is regarded mainly as a secretion product of testicular compartments (Sertoli cells), testicular main ducts and spermatic ducts. Seminal plasma composition as like sperm is different among fish and perhaps reflects not

only an inter- or intra- specific variation (Linhart et al., 1991; Alavi and Cosson, 2006), but also represents inter-strain variations (Koldras et al., 1996). Significant intra- and inter- specific difference has been reported in testicular secretion by Billard et al., (1995b) and Yamazaki and Donaldson, (1968). Interestingly, Lahnsteiner et al., (1993) observed in salmonid fishes that spermatic ducts are different in morphology and histology, but different species represented similar fine structural, histochemical and biochemical features. Therefore, there is major topic for further study to investigate the major organs that control the observed variation in fishes at inter-strain, inter-specific, and intra-specific levels.

Presented data on the ionic composition of the seminal plasma reveals that (a) the Na^+, K^+, and Cl^- ions predominate in seminal plasma, (b) the Cl^-, Na^+, and K^+ contents in sturgeons are lower than teleost fishes, but (c) Na^+/ K^+ ratio is higher in sturgeons than teleost fishes. The osmolality of the seminal plasma of chondrostean fish (sturgeons) is lower than that of teleost fishes. The higher osmolality of the seminal fluid of cyprinid fishes in comparison to that of salmonids (Morisawa, 1985) may be due to higher concentrations of amino acids, peptides, and proteins (Billard and Menezo, 1984).

It has been shown that there is significant variation in seminal plasma composition at different times of the reproductive season in common carp (Kruger et al., 1984; Koldras et al., 1996), landlocked salmon (Piironen, 1985) and rainbow trout (Munkittrick and Moccia, 1987; Billard and Cosson, 1992). Other factors involved in changes of seminal plasma composition are as follows; (a) frequent contamination by urine during stripping (Dreanno et al., 1998; Koldras et al., 1996; Perchec-Poupard et al., 1998; Linhart et al., 2003b), (b) phagositosis of sperm in the testis during degenerative processes during post-spawning when the remaining spermatozoa are resorbed by the spermatic duct epithelium (Billard and Takashima, 1983; Lahnsteiner et al., 1993, 1994), (c) endocrinological parameters such as steroids during spermatogenesis and spermiation (thinning of the semen) (Morisawa et al., 1979; Baynes and Scott, 1985; Marshal and Bryson, 1988; Koldras et al., 1996), feeding regime of broodfish (Dreanno et al., 1998; Dabrowski and Ciereszko, 2001) and (d) different manipulation in spawning induction (Linhart et al., 2003) and artificial reproduction such as stage of anesthesia of broodfish , stripping frequency and time (Piros et al., 2002; Linhart et al., 2003a; Alavi et al., 2005) and origination of broodfish (Piros et al., 2002).

6- Sperm motility

The spermatozoa of most fish species are immotile in the testis and in the seminal plasma (Morisawa, 1985; Billard and Cosson, 1992). K^+ has a major inhibitory factor of Salmonidae (Billard, 1992) and Acipenseridae (Alavi et al., 2004) spermatozoa motility while the inhibition of motility of sperm is due to osmotic pressure in Cyprinidae (Cosson et al., 1991; Billard et al., 1995b) and marine fishes (Morisawa et al., 1999), high or low respectively compared to seminal fluid. Therefore, motility of spermatozoa is induced after release into the aqueous environment during natural reproduction or the diluent during artificial reproduction. It is clear that the motility of sperm is induced due to hypo- and hyper- osmotic pressure in freshwater and marine fishes, respectively (Cosson et al., 1991; Morisawa et al., 1999; Alavi and Cosson, 2006). Cellular studies on the mechanism of sperm motility in fish showed that spermatozoa use external factors as the triggering factor for initiation of the intracellular cascade of events that leads to the initiation of

flagellar beating. In most species, Ca^{2+} influx and K^+ or Na^+ efflux through specific ionic channels cause the changes of membrane potential and eventually lead to cAMP synthesis in the cell signaling for the initiation of sperm motility in specific species such as Salmonidae (Morisawa et al., 1999; Krasznai et al., 2000; Morisawa, 2008). Sperm motility is generated by a highly organized microtubule-based scaffold structured called the axoneme (Inaba, 2003, 2008).

Sperm motility parameters such as beat frequency, ATP content, spermatozoa velocity and the percentage of motile spermatozoa decrease rapidly after triggering the initiation of sperm motility (Billard and Cosson, 1992; Cosson et al., 1999; Alavi and Cosson, 2006). There are several factors affecting sperm motility such as temperature, pH, osmolality, ions as well dilution ratio of prediluted sperm in immobilizing medium or of sperm in activation medium (Billard et al., 1995a,b; Alavi and Cosson, 2005, 2006). Specific studies on sperm motility in terms of flagellar beating and wave parameters have led to a better understanding of the internal mechanics explaining how the movement characteristics of sperm flagella are established (Cosson et al., 2000, 2008a,b; Alavi et al., 2008). Flagellar wave pattern presents also an original feature during the motile phase of fish spermatozoa: it evolves rapidly from fully motile sperm with waves present along the entire flagellum length, to intermediate patterns with waves present only in the proximal part of the flagellum (Cosson, 2008a). The last phase of the motility period is identified by the restriction of the waves to one third or one quarter of the flagellum length leading to a lower and lower efficiency of the sperm translation (Cosson 2008a,b) rapidly followed by the full cessation of movement. This evolution of the wave pattern is paralleled by a decline in flagellar beat frequency during the swimming period, both contributing to a decrease in the swimming ability (Cosson et al., 2000, 2008a,b).

7- Methods for analyzing sperm motility

There are several parameters that can be considered for sperm motility assessments (Cosson, 2008a); (a)velocity of spermatozoa, (b) percentage of motile cells, (c) duration of motility, *i.e.,* time period after activation leading to total immotility of all cells, (d) linearity of track of sperm heads, (e) shape of the flagellar waves and other criteria.

By simple visual inspection it is possible to conclude whether sperm samples are dead or alive. After dilution of sperm in a swimming solution, sperm head movements are observed by regular microscopy. Further, the period needed to reach immotility should also be assessed. Video recording provides a more accurate documentation of the motility and allows subsequently a more precise estimation and can also identify parameters such as velocity by evaluating the head displacement or the shape of the head tracks. Automation using computer-supported analytical tools which recognize the head positions in individual successive frames can be used to reach the percentage of motile cells, the average velocity and other quantifyable parameters, such as the amplitude of head and flagellum undulations. Some more sophisticated technologies allow also in real time to analyze parameters such as velocity of any individual spermatozoon (e.g. Hobson sperm tracker; Van Look and Kime, 2003). Microscopic observations in a dark-field microscope also allow determining the beat frequency (Cosson et al., 1985, 1997). Video recordings with high frequency stroboscopy can also add new dimensions to sperm quality assessment.

Most of the CASA (Computer Assisted Sperm Analysis) systems are designed to obtain information about the head movement: the resolution used to obtain these video images usually does not allow obtaining information about flagella. Sperm trackers (example: Hobson sperm tracker) were successfully used to track fish spermatozoa (Kime et al., 1996), even at the earliest part of the motility period, that is when their velocity is highest (Abascal et al, 2007).

8- Aims of this study

In this thesis, a comparative study was done on biological aspects of sperm in some models of teleostean (*Barbus barbus*, *Perca fluviatilis*, *Esox lucius*) and chondrostean (*Acipenser ruthenus*) fishes with emphasizes on sperm morphology, seminal plasma characteristics and sperm motility.

References

Abascal, F., Cosson, J., Fauvel, C., 2007. Characterization of sperm motility in sea bass: the effect of heavy metals and physicochemical variables on sperm motility. J. Fish Biol. 70, 509–522.

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes: (I) Effects of pH and temperature. Cell Biol. Int. 29, 101–110.

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A., 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction 128, 819–828.

Alavi, S.M.H., Rodina, M., Cosson, J., Psenicka, M., Linhart, O., 2008. Roles of extracellular Ca^{2+} and pH on motility and flagellar wave form parameters in sturgeon spermatozoa. Cybium 32(2), 124–126.

Baccetti, B., Burrini, A.G., Callaini, G., Gibertini, G., Mazzini, M., Zerunian, S., 1984. Fish germinal cells. I. Comparative spermatology of seven cyprinid species. Gamete Res. 10, 373–396.

Barcellos, L.J.G., Wassermann, G.F., Scott, A.P., Woehl, V.M., Quevedo, R.M., Ittzès, I., Krieger, M.H. and Lulhier, F., 2002. Plasma steroid concentrations in relation to the reproductive cycle of cultured male *Rhamdia quelen*. J. Fish Biol. 61, 751–763.

Baynes, S. M., Scott, A.P., 1985. Seasonal variations in parameters of milt production and in plasma concentration of sex steroids in male rainbow trout, *Salmo gairdneri.* Gen. Comp. Endocrinol. 57, 150–160.

Billard, R., 1992. Reproduction in rainbow trout: sex differentiation, dynamics of gametogenesis, biology, and preservation of gametes. Aquaculture 100, 263–298.

Billard, R., Cosson, M.P., 1992. Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122–131.

Billard, R., Menezo, Y., 1984. The amino acid composition of rainbow trout *Salmo gairdneri* seminal fluid and blood plasma: a comparison with carp *Cyprinus carpio*. Aquaculture 41, 341–349

Billard, R., Takashima, F., 1983. Resorption of spermatozoa in the sperm duct of rainbow trout during the post-spawning period. Bull. Jap. Soc. Sci. Fish. 49, 387–392.

Billard, R., Cosson, J., Crim, L.W., Suquet, M., 1995a. Sperm physiology and quality. In: *Brood stock management and egg and larval quality,* Bromage, N.R., Roberts, R.J., (Eds.). Pp. 25–52. Blackwell Science, Amsterdam.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995b. Biology of sperm and artificial reproduction in carp. Aquaculture 124, 95–112.

Billard, R., Fostier, A., Weil, C., Breton, B., 1982. Endocrine control of spermatogenesis in teleost fish. Can. J. Fish. Aquat. 39, 65–79.

Bromage, N.R., Roberts, R.J., 1995. Broodstock management and egg and larval quality. Blackwell Science Ltd. Oxford, 424 pp.

Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquac Int. 12, 69–85.

Cosson, J., 2008a. Methods to analyse the movements of fish spermatozoa and their flagella. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 63–102. Alpha Science Ltd, Oxford, UK.

Cosson, J., 2008b. The motility apparatus of fish spermatozoa. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 281–316. Alpha Science Ltd, Oxford, UK.

Cosson, J., Billard, R., Cibert, C., Dreanno, C., Linhart, O., Suquet, M., 1997. Movements of Fish Sperm Flagella studied by High Speed Videomicroscopy coupled to Computer Assisted Image Analysis. Pol. Arch. Hydrobiol. 44, 103–113.

Cosson, J., Billard, R., Cibert, C., Dreanno, C., Suquet, M., 1999. Ionic factors regulating the motility of fish sperm. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 161 – 186. Cache Rive Press. Vienna IL.

Cosson, J., Billard, R., Redondo-Muller, C., Cosson, M.P., 1991. *In vitro* incubation and maturation of common carp, *C. carpio*, spermatozoa, Bull, Inst, Zool, Acad, Sinica Monograph, 16, 49 – 61.

Cosson, J., Groison, A-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008a. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460–486.

Cosson, J., Groison, A-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008b. Marine fish spermatozoa: racing ephemeral swimmers. Reproduction 136, 277–294.

Cosson, J., Linhart, O., Mims, S., Shelton, W., Rodina, M., 2000. Analysis of motility parameters from paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) spermatozoa. J. Fish Biol. 56, 1348–1367.

Cosson, M.-P., Billard, R., Gatti, J.-L. and Christen, R., 1985. Rapid and quantitative assessment of trout spermatozoa motility using stroboscopy. Aquaculture, 46: 71–75.

Coward, K., Bromage, N.R., Hibbitt, O., Parrington, J., 2002. Gametogenesis, fertilization and egg activation in teleost fish. Rev. Fish Biol. Fish. 12, 33–58.

Dabrowski, K., Ciereszko, A., 2001. Ascorbic acid and reproduction in fish: Endocrine regulation and gamete quality. Aqua. Res. 32, 1–19.

Dreanno, C., Suquet, M., Desbruyeres, E., Cosson, J., Le Delliou, H., Billard, R., 1998. Effect of urine on semen quality in Turbot, *Psetta maxima*; Aquaculture, 169, 247–262.

Grier, H.J., 1981. Cellular organization of the testis and spermatogenesis in fishes. American Zoologist, 21, 345–357.

Inaba, K., 2003. Molecular architecture of the sperm flagella: Molecules for motility and signaling. Zool. Sci. 20, 1043–1056.

Inaba, K., 2008. Molecular mechanisms of the activation of flagellar motility in sperm. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, R., Rafiee, G., (Eds.). Pp. 267–279. Alpha Science Ltd, Oxford.

Jamieson, B.G.M., 1991. *Fish Evolution and Systematics: Evidence from Spermatozoa*. Cambridge University Press, Cambridge. ISBN 0 521 41304 4.

Kime, D.E., Ebrahimi, M., Nysten, K., Roelants, I., Rurangwa, E., Moore, H.D.M., Ollevier, F., 1996. Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish; application to the effects of heavy metals. Aqua. Toxicol. 36, 223–237.

Koldras, M., Loir, M., Maisse, G., Le Gac, F., 1996. Study of the composition of seminal fluid and of sperm motility along the genital tract, during a spawning season in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Living Resour. 9, 337–345.

Krasznai, Z., Marian, T., Izumi, H., Damjanovich, S., Balkay, L., Tron, L., Morisawa, M., 2000. Membrane hyperpolarization removes inactivation of Ca^{2+} channels leading to

Ca^{2+} influx and initiation of sperm motility in the common carp. Proc. Nat. Acad. Sci. 97, 2052–2067.

Kruger, J.C., Smith, G.L., Van Vuren, J.H.J., Ferreira, J.T., 1984. Some chemical and physical characterestics of the semen of *Cyprinus carpio* and *Oreochromis mossambicus*. J. Fish Biol. 24, 263–272.

Lahnsteiner, F., Patzner, R.A., 2008. Sperm morphology and ultrastructure in fish. In: *Fish Spermatology,* Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G., (Eds.). Pp. 1–61. Alpha Science Ltd, Oxford.

Lahnsteiner, F., Patzner, R.A., Weismann, T., 1993. The spermatic ducts of salmonid fishes (Salmonidae, Teleostei). Morphology, histochemistry and composition of the secretion. J. Fish Biol. 42, 79–93.

Lahnsteiner, F., Patzner, R.A., Weismann, T., 1994. The testicular main ducts an spermatic ducts in some cyprinid fishes. II. Composition of the seminal fluid. J. Fish Biol. 44, 459–467.

Le Gac, F., Loir, M., Le Bail, P.Y., Ollitrault, M., 1996. Insulin-like growth factor (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and Sertoli cells. Mol. Reprod. Dev., 44, 23–35.

Linhart, O., Mims, S.D.,Gomelsky, B., Hiott, A.E., Shelton, W.L., Cosson, J., Rodina, M., Gela, D., Bastl, J., 2003a. Ionic and osmolality of paddlefish (*Polyodon spathula,* Acipenseriformes) seminal fluid with motility parameters. Aqua. Int. 11, 357–368.

Linhart, O., Rodina, M., Gela, D., Kocour, M., 2004. Optimalization of artificial propagation in European catfish, *Silurus glanis* L. Aquaculture 235, 619–632.

Linhart, O., Rodina, M., Bastl, J., Cosson J., 2003b. Urinary bladder, ionic composition of seminal fluid and urine with characterization of sperm motility in tench (*Tinca tinca* L.). Journal of Appl. Ichtyol.19, 177–181.

Linhart, O., Slechta, V., Slavik, T., 1991. Fish sperm composition and biochemistry. Bull. Inst. Zool. Acad. Sinica Monograph 16, 285–311.

Loir, M., 1990. Trout steroidogenic testicular cells in primary culture. II. Steroidogenic activity of interstitial cells, Sertoli cells and spermatozoa. Gen. Comp. Endocrinol., 78: 388–398.

Loir, M., Le Gac, F., 1994. Insulin-like growth factor-I and -II binding and action on DNA synthesis in rainbow trout spermatogonia and spermatocytes. Biol. Reprod. 51, 1154–1163.

Mananos, E., Duncan, N., Mylonas, C., 2009. Reproduction and control of ovulation, spermiation and spawning in cultured fish. In: *Methods in Reproductive Aquaculture: Marine and Freshwater Species*, Cabrita, E., Robles, V., Herraez, P., (Eds.). Pp. 3–80. CRC Press Tylor & Francis Group, London.

Marshall, W.S., Bryson, S.E., 1988. Evidence for Cl^- dependent K^+ secretion by the blood-testis barrier of brook trout. Can. J. Zool. 66, 1603–1609.

Marshall, W.S., Bryson, S.E., Idler, D.R., 1989. Gonadotropin stimulation of K^+ secretion and Na^+ absorption by brook trout (*Salvelinus fontinalis*) sperm duct epithelium. Gen. Comp. Endocrinol. 75, 118–128.

Mattei, X., 1991. Spermatozoon ultrastructure and its systematic implications in fishes. Can. J. Zool. 69, 3038 – 3055.

Miura, T., Miura C.I., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem. 28, 181–186.

Miura, T., Highchi, M., Ozaji, Y., Ohta, T., Miura, C., 2006. Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. Proc. Nat. Acad. Sci. 103, 7333–7338.

Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991. Hormonal induction of all stages of spermatogenesis in vitro in male Japanese eel, *Anguilla japonica*. Proc. Natl. Acad. Sci. 88, 5774–5778.

Miura, T., Yamauchi, K., Takahashi. H., Nagahama, Y., 1992. The role of hormones in the acquisition of sperm motility in salmonid fish. J. Exp. Zool. 261, 359–363.

Morisawa, M., 1985. Initiation mechanism of sperm motility at spawning in teleosts. Zool. Sci. 2, 605–615.

Morisawa, M., 2008. Adaptation and strategy for fertilization in the sperm of teleost fish. J. Appl. Ichthyol. 24, 362–370.

Morisawa, S., Morisawa, M., 1986. Acquisition of potential for sperm motility in rainbow trout and chum salmon. J. Exp. Biol. 126, 89–96.

Morisawa, S., Morisawa, M., 1988. Induction of potential for sperm motility by bicarbonate and pH in rainbow trout and chum salmon. J. Exp. Biol. 136, 13–22.

Morisawa, M., Hirano, T., Suzuki, K., 1979. Changes in blood and seminal plasma composition of the mature salmon (*Oncorhynchus keta*) during adaptation to freshwater. Com. Biochem. Physiol. 64, 325–329.

Morisawa, M., Oda, S., Yoshida, M., Takai, H., 1999. Transmembrane signal transduction for the regulation of sperm motility in fishes and ascidians. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Eds.). pp. 149–160. Cache Rive Press. Vienna, IL.

Munikittrick, K.R., Moccia, R.D., 1987. Seasonal changes in the quality of rainbow trout 9Salmo gairdneri) semen: effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma constitutions. Aquaculture 64, 147–156.

Nader, M.R., Miura, T., Ando, N., Miura, C., Yamauchi, K., 1999. Recombinant human insulin-growth factor 1 stimulates all stages of 11-ketotestosterone induced spermatogenesis in the Japanese eel, *in vitro*. Biol. Reprod. 61, 944–947.

Nagahama, Y., 1983. The functional morphology of teleost gonads. In: *Fish Physiology IX*, Hoar, W.S., Randall, D.J., Donaldson, E.M., (Eds). Pp. 223 – 275. Academic Press, New York.

Nagahama, Y., 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217–229.

Parenti, L.R., Grier, H.J., 2004. Evolution and phylogeny of gonad morphology in bony fishes. Integr. Comp. Biol. 44, 333–348.

Patzner, R.A., Lahnsteiner, F., 1999. The accessory organs of the male reproductive system in Mediterranean blennies (Blenniidae) in comparison with those of other small bentic fishes (Tripetrygiidae, Labrisomidae, Chaenopsidae, Clinidae, Gobiidae). In: *Behavior and conservation of littoral fishes*, Almada, V.C., Oliveira, R.F., Goncalves, E.J., (Eds). Pp. 179 – 228. Inst. Sup. Psic. Aplic, Lissabon.

Perchec-Poupard, G., Paxion, C., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1998. Initiation of carp spermatozoa motility and early ATP reduction after milt contamination by urine. Aquaculture 160: 317–328.

Piironen, J., 1985. Variation in the properties of milt from the Finnish landlocked salmon (*Salmo salar m. Sebago* Girard) during a spawning season. Aquaculture 48, 337–350.

Piros, B., Glogowski, J., Kolman, R., Rzemieniecki, A., Domagala, J., Horvath, A., Urbanyi, B., Ciereszko, A., 2002. Biochemical characterization of Siberian sturgeon *Acipenser baeri* and starlet, *Acipenser ruthenus*, milt plasma and spermatozoa. Fish Physiol. Biochem. 26, 289–295.

Rahman, M.S., Takemura, A., Takano, K., 2000. Annual changes in testicular activity and plasma steroid hormones in the golden rabbitfish *Signatus guttatus* (Bloch). Fish. Sci. 66, 894–900.

Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. 26, 43–56.

Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnier, C., Fauvel, C., 1994. Sperm features in turbot (*Scophthalmus maximus*): a comparison with other freshwater and marine fish species. Aquat. Living Resour. 7, 283–294.

Suzuki, K., Kawauchi, H., Nagahama, Y., 1988a. Isolation and characterization of two distinct gonadotropins from chum salmon pititary glands. Gen. Com. Endocrinol. 71, 292–301.

Suzuki, K., Kawauchi, H., Nagahama, Y., 1988b. Isolation and characterization of subunits of two distinct salmon gonadotropins. Gen. Comp. Endocrinol. 71, 302–306.

Thomas, P., Breckenridge-Miller, D., Detweiler, C., 1997. Binding characteristics and regulation of the 17,20ß,21-trihydroxy-4-pregnen-3-one (20ßS) receptor on testicular and sperm plasma membrane of spotted seatrout (*Cynoscion nebulosus*). Fish Physiol. Biochem. 17, 1–6.

Ueda, H., Kambegawa, A., Nagahama, Y., 1985. Involvement of gonadotropin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish *Carassius auratus*. Gen. Comp. Endocrinol. 59, 24–30.

Van Look, K.J.W., Kime, D.E., 2003. Automated sperm morphology analysis in fishes: the effect of mercury on goldfish sperm. J. Fish Biol. 63, 1020–1033.

Vizziano, D., Fostier A., Loir, M., Le Gac, F., 2008. Testis development, its hormonal regulation and spermiation induction in teleost fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 103–139. Alpha Science Ltd, Oxford, UK.

Watanabe, A., Onitake, K., 2008. The regulation of spermatogenesis in fish: Recent cellular and molecular approaches. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 141–160. Alpha Science Ltd, Oxford, UK.

Yamazaki, F., Donaldson, E.M., 1968. The spermiation of gold fish, *Carassius auratus*, as a bioassay for salmon, *O. tschawytscha*, gonadotropin. Gen. Comp. Endocrinol. 10, 383–391.

Yaron, Z., 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129, 49–73.

Chapter 2

Biology of sperm in *Barbus barbus* (Teleostei: Cyprinidae)

Article 1: **Alavi, S.M.H.,** Psenicka, M., Policar, T., Linhart, O., 2008. Morphology and fine structure of *Barbus barbus* (Teleostei: Cyprinidae) spermatozoa. Journal of Applied Ichthyology, 24: 378 – 381.

Article 2: **Alavi, S.M.H.,** Psenicka, M., Rodina, M., Policar, T., Linhart, O., 2008. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Cyprinidae: Teleostei) during the reproductive season. Aquatic Living Resources, 21, 75 – 80.

Article 3: **Alavi, S.M.H.,** Rodina, M., Policar, T., Linhart, O., 2008. Relationships between semen characteristics and body size in *Barbus barbus* L. (Teleostei: Cyprinidae) and effects of ions and osmolality on sperm motility. Comparative Biochemistry and Physiology–Part A, doi: 10.1016/j.cbpa.2009.04.001

Morphology and fine structure of *Barbus barbus* (Teleostei: Cyprinidae) spermatozoa

S. M. Hadi Alavi, Martin Pšenička, Tomáš Policar and Otomar Linhart

University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany 389 25, Czech Republic

Abstract

Morphology and fine structure of *Barbus barbus* L 1758 spermatozoa were studied using scanning (SEM) and transmission (TEM) electron microscopy. The results confirm that spermatozoa exhibit morphological features typical to all teleost fishes. They are differentiated into a head, a midpiece and a flagellum with the typical "9 + 2" pairs of microtubules. Both dynein arms are present in the flagellum. The spermatozoa have spherical nuclei, 4 to 6 mitochondria located in the postnuclear cytoplasmic region and centriolar complex (proximal and distal centrioles). Total length, head width, length of midpiece and length of flagellum were measured to be 56.35 ± 7.42, 1.80 ± 0.06, 0.48 ± 0.14 and 54.30 ± 6.97 μm, respectively. Highly significant linear correlations were observed between posterior and anterior width of midpiece ($P < 0.01$). Principal component analysis (PCA) was used to explore which parameters can explain the individual variation of sperm morphology. About 44% of the total accumulated variance was absorbed by the analysis of the two first components, distinguishing different groups of parameters related to head and midpiece. The lengths of flagellum and head are more isolated; indicating that the individual variation of sperm morphology depends on these two parameters. Comparing the results of this study with information on cyprinids spermatozoa reveals that the number of mitochondria and the length of the flagellum are good characters to characterize spermatozoa of the Cyprinidae in a phylogenetic arrangement.

Key words: Barbus barbus, Flagellum, Mitochondria, Proximal and distal centrioles, Seminal vesicles

1. Introduction

Comparative spermatology has contributed in recent years to understand fish phylogeny. Studies on morphology and fine structure of spermatozoa are considered some of the major subjects in comparative spermatology (Jamieson, 1991; Hara and Okiyama, 1998). The most recent reviews demonstrated that there are differences in the fine structure of sperm at family-specific (Jamieson, 1991; Mattei, 1991) and at subfamily and species-specific level (Lahnsteiner and Patzner, 2008). The fine structure of spermatozoa has been studied in more than 320 species from more than 100 families (Lahnsteiner and Patzner, 2008) and still our understanding of the variability of morphological and their implication for functional phylogeny is fragmentary.

In general, fish sperm is differentiated into a head, a midpiece and a flagellum with the typical 9 + 2 pairs of microtubules. The head contains the nucleus and therefore the parental DNA material. The mitochondria and centriolar complex (proximal and distal centrioles) are located in the midpiece. Mitochondria deliver the energy required for flagellar movement and swimming activity (Jamieson, 1991; Lahnsteiner and Patzner, 2008).

There are few data documented the various biological features of *B. barbus* spermatozoa (Baccetti et al., 1984; Alavi et al., 2008). Baccetti et al., (1984) studied sperm morphology in *B. barbus plebejus* using scanning and transmission electron microscopy, but no quantitative data were presented. Alavi et al., (2008) reported the changes of morphology of and ultrastructure, volume and density of sperm and velocity of spermatozoa derived from *B. barbus* males during the reproductive season.

The objectives of this study were (a) to demonstrate the similarity of the morphological features of *B. barbus* spermatozoa with known cyprinid sperm, (b) to gain specific insight into the un-known ultrastructure through TEM and (c) to identify the intra-specific variability of the studied variables in comparison to closely related species.

2. Materials and methods

Semen of nine 2 year old males (weight 47.2 ± 4.2 g, total length 187.7 ± 7.6 cm) was collected In April 2006. The males were kept in a recirculating system. Water flow, water temperature, oxygen and pH of the system were controlled at 10 L min^{-1}, 21.5 ± 0.5 °C, oxygen 6.8 ± 0.3 mg O_2 L^{-1} and pH 6.8 ± 0.3. Semen was collected with a syringe fitted with a plastic needle. We attempted to avoid its contamination by urine, mucus or blood. Spermiation was not induced in the by hormonal injection. Fish were anesthetized before stripping. Syringe with semen of each male was placed on ice (0 - 2 ^{o}C) and immediately transported to the laboratory for processing.

The sperm of 9 males were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 days at 4 °C (dilution ratio: 1 µl of sperm: 49 µl of fixative), post-fixed for 2 h at 4 °C in osmium tetroxide, washed repeatedly and dehydrated through an acetone series (30, 50, 70, 90, 95 and 100%). Samples for scanning electron microscopy were dehydrated in a critical point dryer pelco CPD 2 (Ted Pella, Inc., Redding, CA), coated with gold under vacuum with scanning electron microscopy coating Unit E5100 (Polaron Equipment Ltd., England) and examined with a JSM 6300 scanning electron microscope (JEOL Ltd., Akishima, Tokyo, Japan), equipped with Sony CCD camera. Samples for transmission electron microscopy were embedded in resin (Polybed 812). A series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Mikrosysteme Gmbh, Austria), double-stained with uranyl acetate and lead citrate and viewed in a JEOL 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at 80 kV. Sperm structures from recorded images were measured using Olympus MicroImage software (version 4.0.1. for Windows).

Data analysis

Results are presented as means ± standard deviation (S.D.). After testing for normality of data using Kolmogrov–Smirnov test, statistical comparisons were made by

employing the Duncan's test for the morphological parameters. Statistical comparisons were considered significantly different at $P < 0.05$ (SPSS 10.0). Principal components analysis (PCA) was used to explore which of the parameters can explain the individual variation of sperm morphology according to Psenicka et al., (2007).

3. Results

Scanning (SEM) and transmission (TEM) electron microscopy confirmed expected sperm features in the common barbel being uniflagellated and differentiated into a head, a midpiece with mitochondria, while having proximal and distal centrioles and a flagellum with the typical structure of "9 + 2" microtubules (Figs. 1 and 2). The sperm is long with a total length 56.35 ± 7.42 μm while the roundish head has the usual width of 1.80 ± 0.06 μm. As the flagellum inserted mediolaterally onto the nucleus, the sperm was asymmetrical, which give the sperm the image of a golf club. The head contains a spherical nucleus, full of compact and homogeneous chromatin. Most of the heads were composed of dense and slightly granular materials which appeared to be homogeneous except for the occasional appearance of vacuoles or vesicles. The postnuclear cytoplasmic region contains 4 to 6 mitochondria. The flagellum length was measured 54.30 ± 6.97 μm and contains a "9 + 2" microtubular structure: both dynein arms are present (Fig. 3).

Morphometric parameters of spermatozoa sampled from 9 males are summarized in Table 1. ANOVA revealed significant differences for most measured parameters except for the posterior width of midpiece (PWM) (Table 2). Posterior and anterior width of midpiece (AWM) exhibited a significant correlation as shown in Fig. 4 ($P < 0.01$).

Principal components analysis (PCA) explained some of the individual variations of sperm morphology. About 44 % of the total accumulated variance was absorbed by the analysis of two first components (Fig. 5), thereby distinguishing the different group characteristics. The first one consists of parameters related to sperm heads (nucleus vesicle (NV) and width of head (HW)). The remaining variables are related to midpiece components (MPL, AMPW and PMPW) and are grouped together. Lengths of the flagellum and head are more isolated, showing that the individual variation of *B. barbus* spermatozoa depends mainly on these two parameters while others are more consistent. The importance of each variable in each component (Table 3) can be assessed as follows: length of the flagellum (FL) having the biggest negative coefficient (-0.47) in the factor 2, but nucleus vesicles (NV) lead to the biggest positive coefficient (0.68). This permits the interpretation that FL has the greatest effect on factor 2, but in the opposite direction of other variables that are positively related such as NV, MPL (0.21) and HW (0.58).

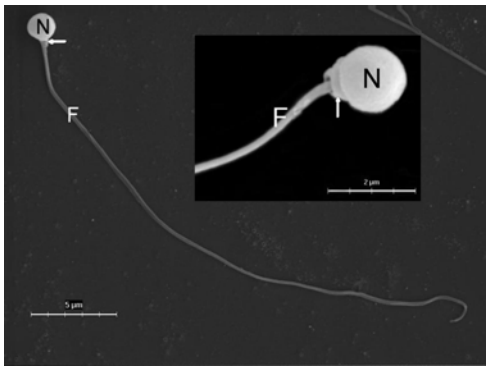

Fig. 1: *Barbus barbus* sperm cell structure using scanning electron microscopy. Nucleus (N), Midpiece (arrow) and flagellum (F).

Table 1: The mean, standard deviation and number of measurement of morphological and ultrastructural parameter of Barbus barbus spermatozoa. Groups with a common superscript did not differ significantly (P > 0.05).

Parameter	Male 1	Male 2	Male 3	Male 4	Male 5	Male 6	Male 7	Male 8	Male 9	Mean
Head length (µm)	1.71(0.09) ab	1.73(0.12) b	1.69(0.10) ab	1.71(0.07) ab	1.71(0.10) ab	1.74(0.09) b	1.70(0.09) ab	1.74(0.11) b`	1.67(0.10) a	1.71(0.10)
Head width (µm)	1.79(0.05) a	1.83(0.06) bc	1.83(0.05) bc	1.78(0.05) a	1.81(3.81) ab	1.80(0.06) ab	1.80(0.05) ab	1.84(0.07) c	1.78(0.06) a	1.80(0.06)
Length of Midpiece (µm)	0.46(0.14) abc	0.46(0.13) abc	0.50(0.12) bc	0.52(0.20) c	0.40(0.11) a	0.45(0.13) ab	0.48(0.13) bc	0.50(0.11) bc	0.49(0.13) bc	0.48(0.14)
Anterior width of Midpiece (µm)	0.80(0.17) ab	0.82(0.19) ab	0.90(0.14) cd	0.81(0.16) ab	0.78(0.17) a	0.79(0.15) a	0.88(0.16) bcd	0.95(0.16) d	0.85(0.13) abc	0.85(0.16)
Posterior width of Midpiece (µm)	0.51(0.13) ab	0.53(0.15) ab	0.55(0.14) ab	0.54(0.13) ab	0.53(0.16) ab	0.51(0.12) a	0.53(0.12) ab	0.59(0.16) b	0.53(0.12) ab	0.53(0.14)
Flagellum length (µm)	45.91(11.12) a	47.64(13.54) a	57.05(4.69) b	55.35(4.34) b	54.79(5.27) b	53.08(3.65) b	55.66(4.16) b	55.50(3.84) b	56.70(10.02) b	54.30(6.97)
Nucleus vesicle (%)	1.74(1.61) d	1.15(0.94) c	0.92(0.80) c	0.30(0.34) a	0.43(0.53) ab	0.48(0.39) ab	0.94(0.69) c	0.74(0.63) bc	1.00(0.68) c	0.84(0.87)
Total length (µm)	48.07(11.11) a	48.39(14.74) a	59.25(4.69) b	57.57(4.36) b	56.39(4.99) b	55.01(3.97) b	57.85(4.16) b	57.59(4.44) b	58.88(10.05) b	56.35(7.42)

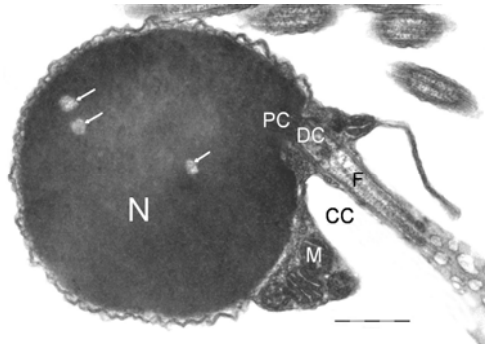

Fig. 2: Sagittal section of the head using transmission electron microscopy showing the distal (DC) and the proximal (PC) centriole. Numerous mitochondria (M) were irregularly dispersed in the cytoplasm. Vesicles (arrows) are present in the nucleus. The flagellum (F) was separated by the cytoplasmic channel (CC).

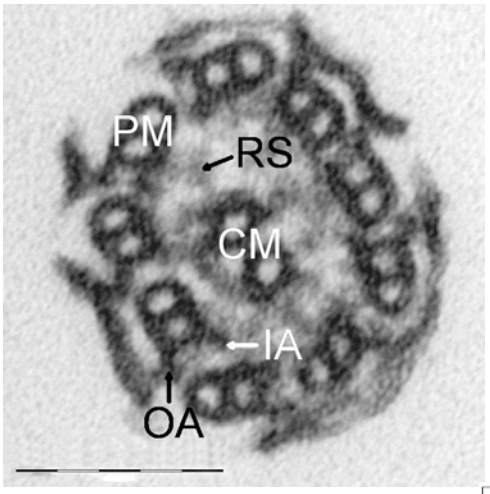

Fig. 3: Cross section of the axoneme transmission electron micrograph showing peripheral doublets of microtubules (PM) and the central pair of microtubules (CM) with radial spoke (RS). The propelling [engines] were the internal (IA) and external dynein arms (OA).

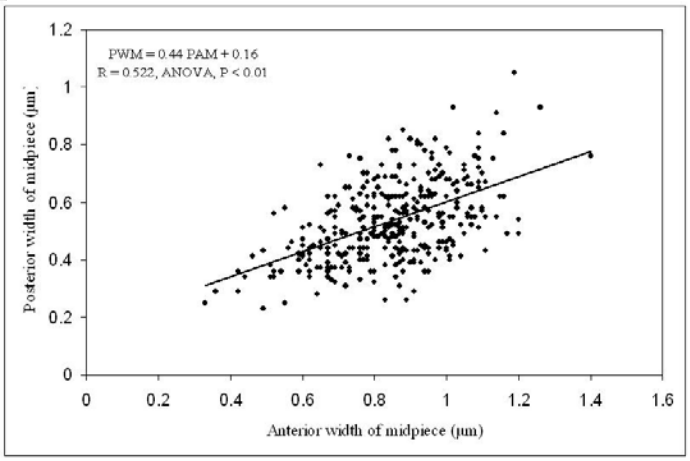

Fig. 4: Linear regression between posterior and anterior width of midpiece of *Barbus barbus* spermatozoa (n= 352 spermatozoa from 9 males).

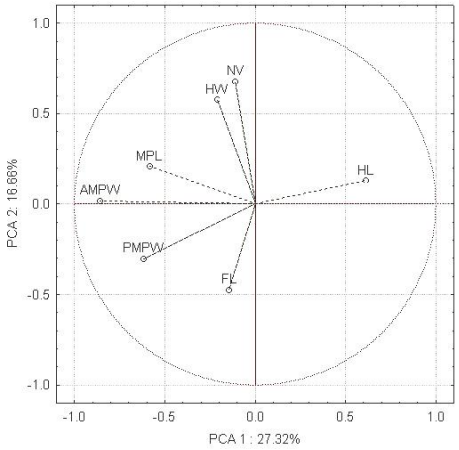

Fig. 5. Ordination diagram of PCA of sperm morphological parameters in *Barbus barbus*. Midpiece length (MPL), Posterior midpiece width (PMPW), Anterior midpiece width (AMPW), Nucleus vesicle (NV), Head length (HL), Head width (HW), Flagellum length (FL).

Table 2: Analysis of variance (ANOVA) for the morphological and ultrastructural parameters of *Barbus barbus* spermatozoa.

Parameter	Sum of Squares	df	Mean Square	F	Sig.
Head length	0.166	8	0.021	2.256	0.023
Head width	0.118	8	0.147	5.040	0.000
Length of Midpiece	0.372	8	0.464	2.471	0.013
Anterior width of Midpiece	0.993	8	0.124	5.065	0.000
Posterior width of Midpiece	0.151	8	0.189	1.013	0.426
Flagellum length	2622.365	8	327.796	7.850	0.000
Nucleus vesicle	44.143	8	5.518	8.857	0.000
Total length	2778.688	8	347.336	7.508	0.000

Table 3: Factor loading on the components extracted by the principal components analysis of all sperm parameters.

Parameter	Factor 1	Factor 2
Head length (HL)	0.614077	0.130321
Head width (HW)	-0.211375	0.576440
Length of midpiece (MPL)	-0.584339	0.208013
Anterior width of Midpiece (AMPW)	-0.858789	0.018713
Posterior width of Midpiece (PMPW)	-0.615680	-0.301297
Flagellum length	-0.143969	-0.473913
Nucleus vesicle	-0.109759	0.676867

4. Discussion

The structure of *B. barbus* spermatozoa is primitive and as expected similar to sperm of other cyprinids. The results also demonstrated high intra-specific variability for some morphometric parameters. Inter- and intra- species variations have also been shown for morphological parameters by other authors (Jamieson, 1991; Hara and Okiyama, 1998). Baccetti et al., (1984) suggested that the mitochondrial number is a good character which can help to classify spermatozoa of Cyprinidae in a phylogenic arrangement. The numbers of mitochondria have been reported with 2, 2 – 3, 2 – 6, 3 – 4, 4, 4 – 6, 5 – 6 and 10 in *Alburnus alburnus*, *Leuciscus cephalus*, *Tinca tinca*, Chondrostoma *toxostoma*, *Leuciscus souffia*, *Barbus barbus plebejus*, *Rutilus rubilio*, *Carassius auratus*, but 4 – 6 in *B. barbus*. The length of the flagellum also showed high differences among Cyprinid fishes. For example, the length of flagellum has been reported 25, 36, 42, 58 and 60 μm in *T. tinca*, *R. rutilus*, *C. toxostoma*, *C. auratus* and *B. barbus plebejus*, however 54 μm in *B. barbus* (Baccetti et al., 1984; Psenicka et al., 2006). Our data indicate length of the flagellum in B. barbus to be close to the latter two species and are therefore in good agreement with anticipated categorization within close related species.

B. barbus spermatozoa have nuclear vesicles, which have been similarly observed in other teleost fish sperm, such as yellow catfish *Pseudobagrus fulvidraco* (You and Lin, 1996), tench *Tinca tinca* (Psenicka et al., 2006). Its function is not yet fully understood. Chen (2000) suggested that they may decrease mechanical stress during vigorous movements, and hence dampen the potential risk for germ plasma injury when sperm

swim incessantly toward the egg micropyle. Nevertheless, no such nuclear vesicles have been reported in other species such as sturgeon spermatozoa, which only contain electron-dense homogeneous chromatin and indicating sturgeons may be considered more primitive than teleost fishes (Psenicka et al., 2007). Although other criteria for such conclusion.

The centriolar complex position can also vary among cyprinid fishes (Baccetti et al., 1984). In *B. barbus*, similar to *B. barbus plebejus* (Baccetti et al., 1984) the proximal centriole is located in a nuclear canal and appears inclined approximately at 80^{o} on the sagittal plane. According to Baccetti *et al.,* (1984) and Psenicka *et al.,* (2006), the proximal centrioles are inclined to distal centrioles under varying angles in cyprinids: 120^{o} in *L. cephalus* and *C. toxostoma*, 125^{o} in *C. auratus*, 110^{o} in *L. souffia* and 140^{o} in *R. rubilio* and *T. tinca*. The distal centriole can also appear almost tangential to the nucleus and may function as a basal body for the flagellum.

Comparing the results of this study with information on cyprinids spermatozoa reveals that the number of mitochondria and the length of the flagellum are good characters to characterize spermatozoa of the Cyprinidae in a phylogenetic arrangement. Apparently, the length of flagellum varies greatly among cyprinids and this fact may help to explain inter-specific differences among spermatozoa of members of this family with subsequent effects on sperm motility behaviour.

Acknowledgements

This work has been supported by GACR no. 524/06/0817, by MSM no. 6007665809, by the Czech Science Foundation (Project No. 523/06/P142) and by the Ministry for Agriculture of the Czech Republic (project No. NAZV QF 3028) and by QL 71305.

References

Alavi, S.M.H., Psenicka, M., Rodina, M., Policar, T., Linhart, O., 2008. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Teleostei: Cyprinidae) during the reproductive season. Aquat. Living Resour. 21, 75–80. .

Baccetti, B., Burrini, A.G., Callaini, G., Gibertini, G., Mazzini, M., Zerunian, S., 1984. Fish germinal cells. I. Comparative spermatology of seven cyprinid species. Gamete Res. 10, 373–396.

Chen, D.Y., 2000. Impregnation biology – impregnation mechanism and procreation engineering. Beijing: Science Press 1-52, 105–138.

Hara, M., Okiyama, M., 1998. An ultrastructural review on the spermatozoa of Japanese fishes. Bull. Ocean Res. Inst., The University of Tokyo 33, 1–138.

Jamieson, B.G.M., 1991. Fish evolution and systematics: evidence from spermatozoa. Cambridge University Press. Cambridge, pp. 230–295.

Lahnsteiner, F., Patzner, R.A., 2008. Sperm morphology and ultrastructure in fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G., (Eds.). Pp. 1–61. Alpha Science Ltd, Oxford, UK.

Mattei, X., 1991. Spermatozoon ultrastructure and its systematic implications in fishes. Can. J. Zool. 69, 3038–3055.

Psenicka, M., Rodina, M., Nebesarova, J., Linhart, O., 2006. Ultrastructure of spermatozoa of tench *Tinca tinca* observation with scanning and transmission electron microscopy. Theriogenology 64, 1355–1363.

Psenicka, M., Alavi, S.M.H., Rodina, M., Gela, D., Nebesarova, J., Linhart, O., 2007. Morphology and ultrastructure of Siberian sturgeon, *Acipenser baerii*, spermatozoa using scanning and transmission electron microscopy. Biol. Cell 99(2), 103–115.

You, Y.L., Lin, D.J., 1996. The ultrastructure of the spermatozoon of the teleost, *Pseudobagrus fulvidraco*. Acta Biol. Exp. Sin. 29, 235–245.

Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Teleostei: Cyprinidae) during the reproductive season

S. M. Hadi Alavi, Martin Pšenička, Marek Rodina, Tomáš Policar, Otomar Linhart

University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany 389 25, Czech Republic

Abstract

Eighteen spermiating males were randomly selected from a hatchery-reared stock and electronically tagged to record changes in their sperm quality parameters (spermatozoa morphology, ultrastructure and motility, ionic composition and osmolality of the seminal plasma, and sperm volume and density) during the spawning season. Stripping was performed at the beginning of March, April and May. The *Barbus barbus* spermatozoon has a head without acrosome, a midpiece with 4-6 mitochondria and proximal and distal centrioles, and a flagellum with the typical 9+2 pairs of microtubules. Apart from posterior width of the midpiece, morphological and ultrastructural parameters changed significantly during the reproductive season; generally by decreasing toward the end of reproductive season. Sperm volume also decreased from 0.42 in March to 0.15 ml in May, and density from 18.81 in March to 12.45 × 10^9 spz ml^{-1} in May. Osmolality (mOsmol kg^{-1}) was 268 ± 4, 276 ± 2 and 268 ± 2 in March, April and May respectively. Chloride, sodium, calcium and potassium ion concentrations (mM) did not show significant differences between March and April (Cl^-: 125.3 *vs.* 120.5, Na^+: 75.7 *vs.* 69.7, Ca^{2+}: 0.4 *vs.* 0.3 and K^+: 84.7 *vs.* 84.0). The percentage of motile spermatozoa at 15 s post activation did not show a significant difference between dates, but the highest spermatozoa velocity at 15 s post activation was observed in April (91.4 ± 3.2 μm s^{-1}) and then decreased significantly towards the end of the reproductive season (80.6 ± 1.9 μm s^{-1} in May). However, lowest spermatozoa velocity was measured in March (70.4 ± 1.9 μm s^{-1}). This study supports the hypothesis that longer spermatozoa swim faster. Within one stripping, velocity and percentage motility decreased significantly with time post activation. In conclusion, changes observed in *B. barbus* sperm parameters during the reproductive season, suggest there is association between such changes and spermatozoal aging processes.

Key words: Seminal plasma, Sperm, Motility, Morphology of spermatozoa, *Barbus barbus*

1. Introduction

Broodfish management is a key factor determining gamete quality and hence the success of artificial reproduction (Billard et al., 1995; Alavi et al., 2008a,b). Gamete quality also varies during the spawning season in species with annual spawning cycles

(Buyukhatipoglu and Holtz, 1984; Piironen, 1985; Koldras et al., 1996; Rideout et al., 2004; Babiak et al., 2006). Consequently, changes can occur the in qualitative and quantitative characteristics of sperm over the reproductive season.

Sperm morphology, density, volume, motility and fertilizing capacity, as well as composition and osmolality of the seminal plasma, are parameters commonly measured to assess sperm quality in fish (Billard et al., 1995; Linhart et al., 2000; Rurangwa et al., 2004; Alavi and Cosson, 2006). Evaluation of sperm quality could provide information allowing aquaculturists to determine the optimal time for sperm collection, and to devise optimal handling and storage protocols for sperm used in artificial fertilization (Billard et al., 1995; Linhart et al., 2004).

The common barbel, *Barbus barbus* (Linnaeus, 1758) is an endangered species in Europe due to overfishing, poaching and environmental degradation. Such degregation may take the form of the accumulation of pollutants in sediments or the damming of rivers, rendering them unsuitable for migration and reproduction (Lusk, 1996; Lusk et al., 1998; Fiala and Spurny, 2001). A few studies have already been made on the development of artificial breeding and cultivation of common barbel in hatcheries (Poncin et al., 1987; Philippart et al., 1989; Poncin, 1989; Policar et al., 2007).

The aim of present study was to investigate changes in sperm morphology, volume, density and motility, and the osmolality and ionic composition of the seminal plasma during the reproductive season.

2. Materials and methods

2.1. Experimental design, source of fish and collection of sperm

Seed was obtained from an artificial reproduction of natural broodfish captured in the Doubrava River (49° 54′ N and 15° 30′ E) (Policar et al., 2007). The fertilized eggs were transported to the hatchery where hatched larvae were reared in uniform controlled conditions at a density of 100 fish in a recirculating system and fed 78% commercial diet (Karpico™ 33% crude protein and 6% fat) containing 22-44 % frozen chironomid larvae. Total daily food rations were 1.5% of fish total body weight. The food was distributed by automatic feeders during daylight hours. The environmental conditions were maintained at: water temperature 21.5 ± 0.5 °C, oxygen 6.8 ± 0.3 mg O_2 L^{-1} and pH 6.8 ± 0.3.

Eighteen spermiating males were randomly selected from the stock and electronically tagged for use in recording changes in sperm quality parameters during the spawning season. Stripping was carried out at the beginning of March, April and May. In our hatchery conditions, the males produced sperm from mid-February until mid-May. Sperm was collected with a syringe fitted with a plastic needle. We attempted to collect all the sperm at each stripping and to avoid its contamination with urine, mucus or blood. A syringe of sperm from each male was placed on ice (4 ^{o}C) and immediately transported to the laboratory for analyses.

2.2. Sperm Morphology

The non-activated sperm of 7 males were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6) for 2 days at 4 °C (dilution ratio: 1 µl of sperm: 49 µl of

fixative), post-fixed for 2 h at 4 °C in osmium tetroxide, washed repeatedly and dehydrated through an acetone series (30, 50, 70, 90, 95 and 100%). Samples for scanning electron microscopy were dehydrated in a critical point dryer (Pelco CPD 2 ,Ted Pella, Inc., Redding, CA), coated with gold under vacuum with scanning electron microscopy coating Unit E5100 (Polaron Equipment Ltd., England) and examined with a JSM 6300 scanning electron microscope (JEOL Ltd., Akishima, Tokyo, Japan), equipped with a Sony CCD camera. Samples for transmission electron microscopy were embedded in resin (Polybed 812). A series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Mikrosysteme Gmbh, Austria), double-stained with uranyl acetate and lead citrate, and viewed in a JEOL 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at 80 kV. Sperm structures were measured from recorded images using Olympus MicroImage software (version 4.0.1. for Windows).

2.3. Sperm volume and density

Sperm volume and density were measured and expressed in ml and billions of sperm per ml, respectively. Sperm density was determined by counting, following the method of Caille et al., (2006): the sperm was diluted 1000 times with 0.7% NaCl, a drop was placed onto a haemocytometer (depth 0.1 mm), a coverslip was added and the sperm were then left for 10 min to allow sedimentation before counting numbers in 16 cells.

2.4. Ionic composition and osmolality of the seminal plasma

Sperm samples were centrifuged initially at 3000 rpm for 3 min followed by 10 min at 10 000 rpm. The supernatant seminal plasma was stored frozen at -80^{o} C until analysis. Chloride, potassium, and sodium ions were measured using Ion Selective Electrodes, ISE, (Bayer Healthcare, New York, USA) by indirect simultaneous measurement. Calcium concentration was measured by flame photometry. The osmolality of the seminal plasma was measured using a Vapour Pressure Osmometer (Wescor, USA).

2.5. Motility of spermatozoa

Spermatozoa velocity (μm s^{-1}) and percentage of motile spermatozoa after activation (%) were measured using dark-field microscopy (200× magnification). For measurement of spermatozoa motility, sperm was diluted in Tris-HCl 30 mM, pH 8.0 at a 1:1000 dilution ratio. To avoid sperm sticking to the slide, 0.1 % BSA was added. Spermatozoa motility was analyzed from a video recording taken after activation with a 3 CCD video camera (Sony DXC-970MD, Japan) mounted on a dark-field microscope (Nikon Optiphot 2, Japan). The successive positions of sperm heads were measured from five successive video frames for each sperm using a video recorder (Sony SVHS, SVO-9500 MDP, Japan), and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Analysis of spermatozoa motility was carried out in triplicate for each sample. Sperm motility was measured in the same males from which samples had been collected for electron microscopy studies.

2.6. Data analysis

All mean values represent mean ± standard error of mean (SEM). Normality of data was tested with Kolmogrov–Smirnov tests and statistical comparisons then made with Duncan's tests using SPSS 10.0 software. Statistical comparisons were considered significantly different at $p < 0.05$.

3. Results

3.1. Sperm morphology

Scanning and transmission electron microscopy showed that the spermatozoon of common barbel is uniflagellated and differentiated into a head without acrosome, a midpiece with 4-6 mitochondria, proximal and distal centrioles and a flagellum with the typical 9+2 pairs of microtubules (Fig. 1). Changes in morphological and ultrastructural parameters of spermatozoa are summarized in Table 1, these generally first increased, then decreased significantly towards the end of the reproductive season. Percentage of nucleus vesicles decreased significantly into the reproductive season though. The shortest and longest spermatozoa (both flagellar and total) were observed in March and April respectively.

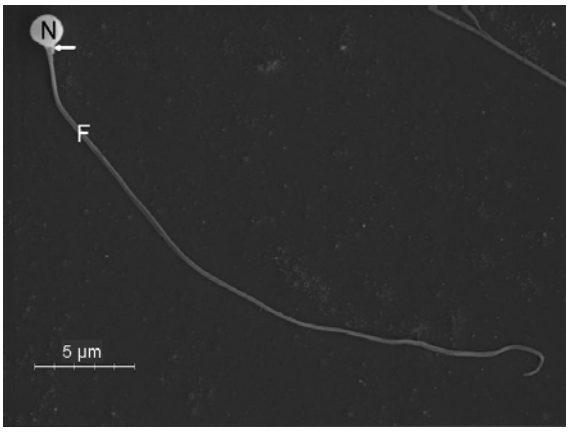

Fig. 1: Structure of *B. barbus* spermatozoa using scanning electron microscopy. Nucleus (N), Midpiece (arrow) and flagellum (F).

3.2. Sperm volume and density

Sperm volume showed a significant decrease towards the end of the spawning season (Fig. 2a). The highest and lowest sperm volumes were recorded in March and May, respectively. In general, sperm density also decreased towards the end of the spawning season (Fig. 2b). However, no significant difference was observed between April and May.

3.3. Ionic composition and osmolality of the seminal plasma

Recordings of ionic composition and osmolality of the seminal plasma are shown in Table 2. No significant differences were observed over the period studied, although the highest osmolality was observed in April (277 ± 3 mOsmol kg^{-1}).

Table 1: Changes in morphological and ultrastructural parameters of *Barbus barbus* spermatozoa during the reproductive season. Different letters indicate statistically significant differences among stripping times ($p < 0.05$).

Parameter	Time of stripping	Number of spermatozoa	Mean ± SEM (µm)
Total length	March	269	48.30 ± 0.37^{a}
	April	274	56.35 ± 0.45^{c}
	May	443	53.65 ± 0.21^{b}
Head length	March	324	1.64 ± 0.01^{a}
	April	352	1.71 ± 0.01^{b}
	May	504	1.62 ± 0.01^{a}
Head width	March	325	1.74 ± 0.01^{b}
	April	352	1.80 ± 0.01^{c}
	May	504	1.72 ± 0.02^{a}
Length of midpiece	March	316	0.48 ± 0.01^{b}
	April	351	0.48 ± 0.01^{b}
	May	504	0.42 ± 0.01^{a}
Midpiece anterior width	March	318	0.87 ± 0.01^{c}
	April	351	0.85 ± 0.01^{b}
	May	504	0.82 ± 0.01^{a}
Midpiece posterior width	March	316	0.52 ± 0.01^{a}
	April	351	0.53 ± 0.01^{a}
	May	504	0.51 ± 0.01^{a}
Flagellar length	March	384	46.52 ± 0.3^{0}
	April	338	54.30 ± 0.38^{c}
	May	662	51.67 ± 0.18^{b}
Nucleus vesicles (per spz)	March	247	1.6 ± 0.2^{b}
	April	280	0.8 ± 0.1^{a}
	May	303	0.8 ± 0.0^{a}

Table 2: Ionic composition (mM) and osmolality (mOsmol kg^{-1}) of the seminal plasma of *B. barbus* during the reproductive season. No parameters showed significant differences among stripping times. Numbers in parentheses indicate the number of samples analysed,

Seminal plasma parameters	March	April	May
Osmolality	268 ± 4 (15)	276 ± 2 (18)	268 ± 2 (12)
Chloride	125.3 ± 1.2 (7)	120.5 ± 3.0 (4)	n.d.
Sodium	75.7 ± 1.8 (7)	69.7 ± 4.0 (4)	n.d.
Calcium	0.4 ± 0.0 (7)	0.3 ± 0.1 (4)	n.d
Potassium	84.7 ± 2.8 (7)	84.0 ± 4.9 (4)	n.d.

n.d.: not determined

3.4. Motility of spermatozoa

The highest spermatozoa velocity was observed in April at 15 s post activation (Fig. 3). For the same stripping time, spermatozoa velocity decreased significantly as a

function of time post activation. There were no significant differences in the percentage of motile spermatozoa at 15 and 30 s post activation.

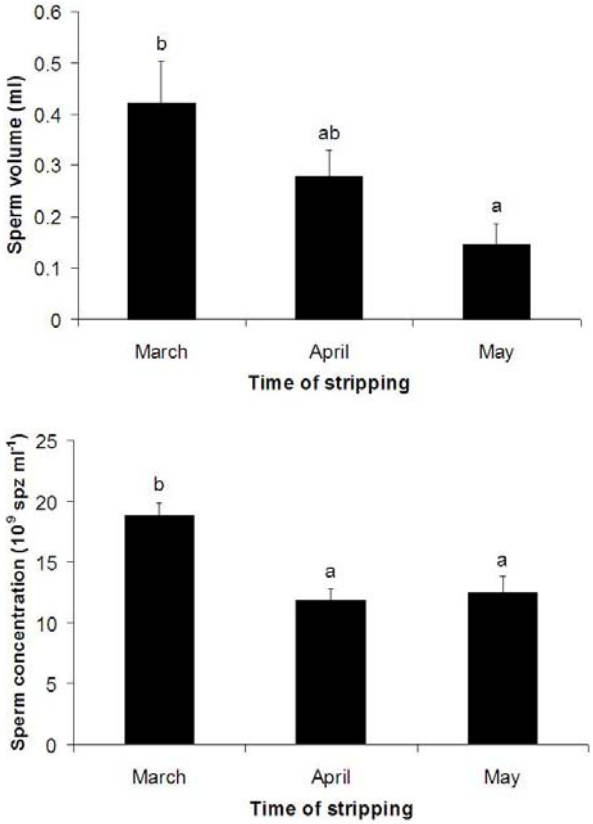

Fig. 2: Changes in sperm volume (a), and sperm density (b) in *B. barbus* during the reproductive season. Bars with different superscripts indicate statistically significant differences among stripping times ($p < 0.05$).

Discussion

To our knowledge, this is the first study showing the changes in morphological and ultrastructural parameters of *B. barbus* spermatozoa during the reproductive season using electron microscopy techniques. The changes observed in morphological and ultrastructural parameters illustrate an aging phenomenon in *B. barbus* sperm towards the end of the reproductive season. The aging of spermatozoa has been reported to bring about a diversity of changes in other fish species, affecting morphology, seminal plasma composition, sperm density, spermatozoal ATP content and metabolism, sperm motility and fertilizing ability (Billard et al., 1993; Suquet et al., 1998; Dreanno et al., 1999; Babiak et al., 2006). However, the mechanisms regulating development of spermatozoa during the reproductive season are not well understood. The percentage of nucleus vesicles in *B. barbus* spermatozoa decreased significantly towards the reproductive season (Table 1), suggesting that these structures might have developed due to dehydration of nuclear material during spermatogenesis as observed by Psenicka et al., (2006).

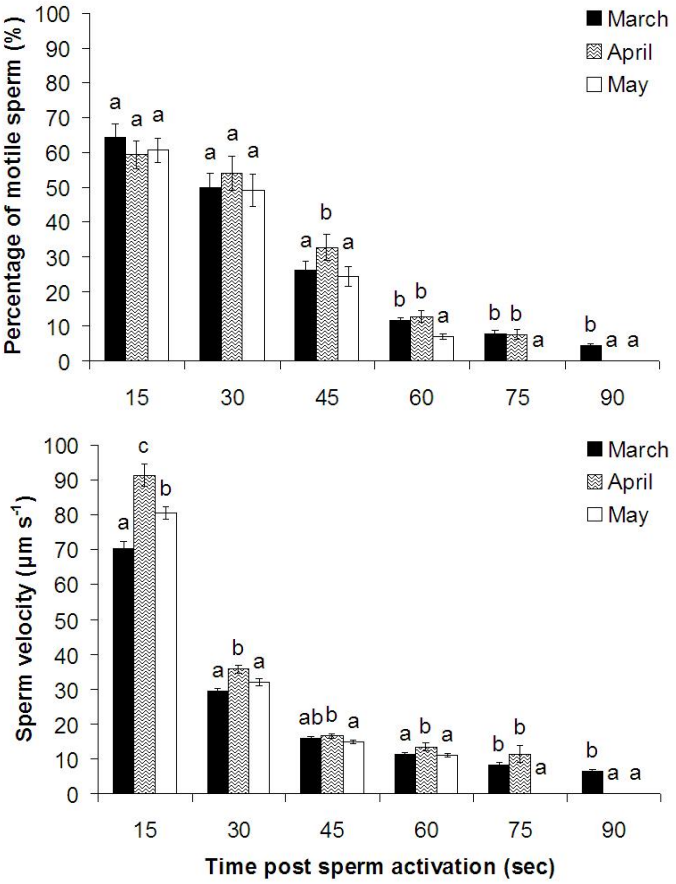

Fig. 3: Changes in sperm motility parameters: spermatozoa velocity (a) and percentage of motile spermatozoa (b) in *B. barbus* during the reproductive season. The velocity values correspond to motile sperm cells. Bars with different superscripts indicate statistically significant differences among strippings for the same time post sperm activation ($p < 0.05$).

Sperm volume changed during the reproductive season, decreasing towards the end of the spawning period. In landlocked salmon (*Salmo salar m. sebago* Girard) however, sperm volume increase has been reported during the reproductive season (Piironen, 1985). Comparisons are made difficult by endocrinological events that regulate spermiation and milt hydration (Mylonas et al., 1997; Vermeirssen et al., 2000). Sperm density has been shown to increase during the spawning season for turbot (Suquet et al., 1998) and Atlantic salmon (Piironen, 1985), but to decrease during the spawning season for tench (Zuromska, 1981), rainbow trout (Buyukhatipoglu and Holtz, 1984; Munkittrick and Moccia, 1987), sea bass and sea bream (Kara and Labed, 1994). In *B. barbus*, sperm density decreased towards the end of the reproductive season. The non-significant increase of sperm density in May compared to April is probably due to reduced sperm dilution rather than increased sperm production (Clemens and Grant, 1965; Shangguan and Crim, 1999).

No significant changes were observed in terms of the seminal plasma ionic composition and osmolality in this study. However, it has been shown that there is significant variation in seminal plasma composition at different times of the reproductive season in common carp (Koldras et al., 1996), landlocked salmon (Piironen, 1985), rainbow trout (Munkittrick and Moccia, 1987), Persian sturgeon (Alavi et al., 2006) and cod (Suquet et al., 2005). In fishes, several factors have been reported that regulate the composition of seminal plasma (see reviews by Ciereszko et al., 2000; Ciereszko, 2008;

Alavi et al., 2008b) including: (a) hormonal mechanisms regulating the spermiation (thinning of the sperm) during the reproductive season; (b) the phagocytosis of sperm in the testis during degenerative processes in post-spawning periods, when any remaining spermatozoa are resorbed by the spermatic duct epithelium; (c) the feeding regime of the broodfish; and (d) the many differing strategies used to induce spawning artificially, such as the duration and intensity of broodfish anaesthesia, stripping frequency and time, and broodfish origin.

In this study, no significant changes were observed in the percentage of motile sperm at 15 s post activation, results similar to those reported in *Gadus morhua* (Suquet et al., 2005) and several cyprinids (Belova, 1981). However, decreases in sperm motility have been documented in several fish species such as rainbow trout (Munkittrick and Moccia, 1987), turbot (Suquet et al., 1998) and haddock (Rideout et al., 2004) as the reproductive season progressed. Differing results on changes of sperm motility over the reproductive season may be due to changing endocrine performance involved in sperm maturation (Rideout et al., 2004; Alavi et al., 2008b). Spermatozoa velocity did however vary significantly during the reproductive season in the present study. The highest and lowest sperm velocities were observed when the spermatozoa had the longest and shortest flagellar lengths respectively. On the other hand, no changes were recorded in terms of the ionic contents and osmolality of the seminal plasma. Therefore, significant changes in spermatozoa velocity may be due to ATP contents of the spermatozoa or depend on their structure (flagellar length). In Atlantic salmon, Vladic et al., (2002) demonstrated that longer spermatozoa have more ATP available and greater fertilization success. Gage et al., (2004) also noted that males with higher spermatozoa velocities fertilized a greater proportion of eggs. Taken together, this suggests that the longest spermatozoa swim the fastest and that energy is used to increase spermatozoa velocity. Here we used buffered distilled water to induce sperm motility. Further studies we made showed that *B. barbus* spermatozoa need higher osmolality of the activation medium (containing 100 mM Na^+ and or sucrose with osmolality 175 - 200 mOsmol kg^{-1}) to show 100% motility.

Acknowledgements

This work was supported by GACR no. 524/06/0817, MSM no. 6007665809, the Czech Science Foundation (Project No. 523/06/P142) and by the Ministry for Agriculture of the Czech Republic (project No. NAZV QF 3028). The authors wish to thank the staff of the fish culture station of RIFCH, USB for maintaining the broodfish for this research.

References

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G., 2008a. *Fish Spermatology*. Alpha Science Ltd, Oxford, UK. ISBN 978 1 84265 369 2.

Alavi, S.M.H., Cosson, J., Kazemi, R., 2006. Semen characteristics in *Acipenser persicus* in relation to sequential stripping. J. Appl. Ichthyol. 22 (Suppl. 1), 400–405.

Alavi, S.M.H., Linhart, O., Coward, K., Rodina, M., 2008b. Fish Spermatology: Implication for aquaculture management. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 397–460. Alpha Science Ltd, Oxford, UK.

Babiak, I., Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 65, 1587–1604.

Belova, N.V., 1981. The ecological and physiological peculiarities of sperm in pond cyprinids. Communication I. Production and ecological and physiological peculiarities of the sperm of cyprinids. J. Ichthyol. 21, 90-102.

Billard, R., Cosson, J., Crim, L.W., 1993. Motility of fresh and aged halibut sperm. Aquat. Living Resour. 6, 67–75.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995. Biology of sperm and artificial reproduction in carp. Aquaculture 124, 95–112.

Buyukhatipoglu, S., Holtz, W., 1984. Sperm output in rainbow trout (*Salmo gairdneri*). Effect of age, timing, and frequency of stripping and presence of females. Aquaculture 37, 63–71.

Caille, N., Rodina, M., Kocour, M., Gela, D., Flajshans, M., Linhart, O., 2006. Quantity, motility and fertility of tench *Tinca tinca* (L.) sperm in relation to LHRH analogue and carp pituitary treatments. Aquac. Int. 14, 75–87.

Ciereszko, A., 2008. Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity and cryopreservation success in fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 215–240. Alpha Science Ltd, Oxford, UK.

Ciereszko, A., Glogowski, J., Dabrowski, K., 2000. Biochemical characteristics of seminal plasma and spermatozoa of freshwater fishes. In: Cryopreservation of Aquatic Species, Tiersch, T.R., Mazik, P.M., (Eds.). Pp. 20–48. World Aquaculture Society, Baton Rouge, Louisiana.

Clemens, H.P., Grant, F.B., 1965. The seminal thinning response of carp, *Cyprinus carpio*, and rainbow trout, *Salmo gairdneri* after injections of pituitary extracts. Copeia 2, 174–177.

Dreanno, C., Cosson, J., Suquet, M., Dorange, G., Fauvel, C., Cibert, C., Billard, R., 1999. Effects of osmolality, morphology and intracellular nucleotid content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. J. Reprod. Fertil. 116, 113–125.

Fiala, J., Spurny, P., 2001. Intensive rearing of the common barbel (*Barbus barbus* L.) larvae using dry starter feeds and natural diet under controlled conditions. Czech J. Anim. Sci. 7, 320–326.

Gage, M.J.G., MacFarlane, C.P., Yeates, S., Ward, R.G., Seare, J.B., Parker, G.A., 2004. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14, 44-47.

Kara, M.H., Labed, S., 1994. Evolution des caractéristiques du sperme de *Sparus aurata* et *Dicentrarchus labrax* au cours d'une saison de reproduction. Cah. Biol. Mar. 35, 281–288.

Koldras, M., Loir, M., Maisse, G., Le Gac, F., 1996. Study of the composition of seminal fluid and of sperm motility along the genital tract, during a spawning season in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Living Resour. 9, 337–345.

Linhart, O., Mims, S.D., Gomelsky, B., Hiott, A.E., Shelton, W.L., Cosson, J., Rodina, M., Gela, D., 2000. Spermiation of paddlefish (*Polyodon spathula*) stimulated with injection of LHRH analogue and carp pituitary extract. Aquat. Living Resour. 13. 1–6.

Linhart, O., Rodina, M., Gela, D., Kocour, M., 2004. Optimalization of artificial propagation in European catfish, *Silurus glanis* L. Aquaculture 235, 619–632.

Lusk, S., 1996. Development and status of populations of *Barbus barbus* in the waters of the Czech Republic. Folia Zool. 45 (Suppl. 1), 39–46.

Lusk, S., Luskova, V., Halacka, K., Slechta, V., Slechtova, V., 1998. Trends and production of a fish communities of the barbel zone. Folia Zool. 47 (Suppl. 1), 67–72.

Munikittrick, K.R, Moccia, R.D., 1987. Seasonal changes in the quality of rainbow trout (*Salmo gairdneri*) semen: effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma constitutions. Aquaculture 64, 147–156.

Mylonas, C.C., Gissis, A, Magnus, Y., Zohar, Y., 1997. Hormonal changes in male white bass (*Morone chrysops*) and evaluation of milt quality after treatment with a sustained-release GnRHa-delivery system. Aquaculture 153, 301–313.

Piironen, J., 1985. Variation in the properties of milt from the Finnish landlocked salmon (*Salmo salar m. sebago* Girard) during a spawning season. Aquaculture 48, 337–350.

Philippart, J.C., Melard, C., Poncin, P., 1989. Intensive culture of the common barbel, *Barbus barbus* (L.) for restocking. In: *Aquaculture – a biotechnology in progress*, De Pauw, N., Jaspers, E., Ackefors, H., Wilkins, N. (Eds.). Pp. 483 – 491. Proc. Int. Conf. Aquaculture Europe'87, 2-5 June 1987, Bredene, Belgium. European Aquaculture Society.

Policar, T., Kozak, P., Hamackova, J, Lepicova, A., Musil, J., Kouril, J., 2007. Effects of short-time *Artemia* spp. feeding in larvae and different rearing environments in juveniles of common barbel (*Barbus barbus*) on their growth and survival under intensive controlled conditions. Aquat. Living Resour. 20, 175–183.

Poncin, P., 1989. Effects of different photoperiods on the reproduction of the barbel, *Barbus barbus* (L.) reared at constant temperature. J. Fish Biol. 35, 395–400.

Poncin, P., Melard, C., Philippart, J., 1987. Use of temperature and photoperiod in the control of the reproduction of 3 European cyprinids: *Barbus barbus* (L.) *Leuciscus cephalus* (L.) and *Tinca tinca* (L.), reared in captivity. Bull. Fr. Pêche Piscic. 304, 1–12.

Psenicka, M., Rodina, M., Nebesarova, J., Linhart, O., 2006. Ultrastructure of spermatozoa of tench *Tinca tinca* observation with scanning and transmission electron microscopy. Theriogenology 64, 1355–1363.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. J. Fish Biol. 65, 319–332.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. Measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1–28.

Shangguan, B., Crim, L.W., 1999. Seasonal variation in sperm production and sperm quality in male winter founder, *Pleuronectes americanus*: the effects of hypophysectomy, pituitary replacement therapy, and GnRH-a treatment. Mar. Biol. 134, 19–27.

Suquet, M., Dreanno, C., Dorange, G., Normant, Y., Quemener, L., Gaignon, J.L., Billard, R., 1998. The ageing phenomenon of turbot, *Scophthalmus maximus*, spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization and storage capacities. J. Fish Biol. 32, 31–41.

Suquet, M., Rouxel, C., Cosson, J., Severe, A., Quemener, L., Fauvel, C., 2005. Changes in atlantic cod (*Gadus morhua*) sperm quality with time. In: *Larvi 2005*, Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloss, P., (Eds.). Pp. 503–505. European Aquaculture Society, Spec. Publication.

Vermeirssen, E.L.M., Mazorra de Quero, C., Shields, R., Norberg, B., Kime, D.E., Scott, A.P., 2004. Fertility and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*) in relation to dose and timing of gonadotropin-releasing hormone agonist implant. Aquaculture 230, 547–567.

Vladic, T.V., Afzelius, B.A., Bronnikov, G.E., 2002. Sperm quality as reflected through morphology in salmon alternative life histories. Biol. Reprod. 66, 98–105.

Zuromska, H., 1981. Effect of different thermal regimes on reproductive cycles of tench (*Tinca tinca*). Part VI. Estimation of milt quality. Pol. Arch. Hydrobiol. 28, 229–241.

Relationships between semen characteristics and body size in *Barbus barbus* L. (Teleostei: Cyprinidae) and effects of ions and osmolality on sperm motility

S. M. Hadi Alavi, Marek Rodina, Tomáš Policar, Otomar Linhart

Research institute of Fish Culture and Hydrobiology, University of South Bohemia, Vodnany 389 25, Czech Republic

Abstract

The objectives of the present study were to determine the relationships among length and weight of males, sperm volume, spermatozoa concentration, total number of spermatozoa, ionic contents and osmolality of seminal plasma in *Barbus barbus*. The effect of osmolality on sperm motility parameters after activation in NaCl, KCl, or sucrose solutions was also examined. There were significant correlations between spermatozoa concentration – length (R= +0.7) and – weight (R= +0.8) of males. No significant correlations were observed between the total number of spermatozoa, sperm volume, and length and weight of males. Seminal plasma osmolality was higher when the total number of spermatozoa (R= +0.6) and sperm volume (R= +0.6) were higher. Sperm motility and velocity was positively correlated with osmolality (R= +0.5). The correlation between sperm motility and K^+ was negative (R= 0.5), but positively correlated with Ca^{2+} (R= 0.8), Na^+ (R= 0.8), and Cl^- (R= 0.8). There was a rapid decrease ($P < 0.05$) in sperm motility parameters after sperm activation. Just after sperm activation, beating waves propagated along the full length of flagella. At latter stages post sperm activation, the waves appeared only in proximal part of the flagellum. The highest spermatozoa velocity and percentage of motility were observed at 215 – 235 mOsmol kg^{-1} in NaCl, KCl or sucrose. The tip of the flagellum became curled into a loop shape which shortened the flagellum after activation of sperm in distilled water. *B. barbus* sperm is very similar to that of other cyprinids in terms of ionic contents and osmolality of the seminal plasma, mechanism of sperm activation and behavior and motility of sperm during swimming period.

Key words: Motility, Velocity, Flagellum, Sperm volume, Spermatozoa concentration, Total number of spermatozoa, Sperm trajectory

1. Introduction

The barbel, *Barbus barbus* L. (Teleostei: Cyprinidae) is a freshwater species, which is abundantly distributed throughout western and central Europe (Poncin, 1989). They are considered as an endangered species in Europe mainly due to environmental degradation such as, the accumulation of pollutants in sediments and damming of rivers, rendering these waterways unsuitable for reproduction (Lusk, 1996). Re-stocking of yearlings of artificial reproduction can improve the natural populations if attention is paid to preserving intra-specific genetic diversity (Slechtova et al., 1998). In this regard, sperm management can become a vial tool for increasing artificial reproduction/fertilization success in fish farms (Billard et al., 1995a).

Fish spermatozoa are immotile in the testis, but gain potentiality for activation during transfer to the sperm duct. The physiological changes that occur after transfer is controlled by an endocrinological system, which regulate spermatogenesis and spermiation (Billard et al., 1995a,b; Nagahama, 1994). Seminal plasma produced by the sperm duct provides an ionic environment that maintains the viability of spermatozoa after their release from the testes (Ciereszko, 2008). It has been already shown in the literature that there are several correlations between seminal plasma composition and sperm motility in some species; Atlantic salmon, *Salmo salar* (Hwang and Idler, 1969), common carp, *Cyprinus carpio* (Kruger et al., 1984), bleak, *Alburnus alburnus* (Lahnsteiner et al., 1996), rainbow trout, *Oncorhynchus mykiss* (Lahnsteiner et al., 1998), Persian sturgeon, *Acipenser persicus* (Alavi et al., 2004a) and chinook salmon, *Oncorhynchus tshawytscha* (Rosengrave et al., 2009). In freshwater species, matured spermatozoa need a hypo-osmotic shock for triggering initiation of sperm motility (Morisawa et al., 1999). Moreover, there are several factors that affect sperm motility such as pH, temperature, ions and osmolality (Cosson et al., 1999; Morisawa et al., 1999; Alavi and Cosson, 2006). Studying the effects of these factors on sperm quality can help establish good activation and/or immobilizing media for improving either artificial fertilization or cryopreservation protocols.

Past research has examined sperm morphology (Alavi et al., 2008a) and changes in sperm quality during the reproductive season (Alavi et al., 2008b) in the barbel. Finding reported that apermatozoon of the barbel have a total and flagellar length 56 and 54 μm, respectively. The barbel sperm was shown to be differentiated into a head (without the presence of an acrosome), a midpiece (containing 4 – 6 mitochondria and proximal and distal centrioles) and a flagellum with the typical "9 + 2" pairs of microtubules (Alavi et al., 2008a). Morphological comparison with other cyprinids spermatozoa reveals that the number of mitochondria and the length of the flagellum are good features to distinguish spermatozoa of the Cyprinidae into a phylogenetic arrangement. Neither osmolality nor ionic contents of seminal plasma differed significantly during reproductive season. However, sperm velocity and motility changed during spawning season. Currently, no work has been done to characterize the effects of ions and osmolality on motility of sperm as well physiological relationships between sperm motility and seminal plasma ionic composition and osmolality.

In this paper, the relationships among length and weight of males, sperm volume, spermatozoa concentration, total number of spermatozoa, ionic contents and osmolality of the seminal plasma and sperm motility are investigated. This is the first study which (a) illustrates dynamics of sperm motility after activation and (b) examines the effects of osmolality on sperm motility parameters after activation in NaCl, KCl, or sucrose solutions. This information will provide a better understanding of the mechanism regulating sperm activation and sperm behaviour.

2. Materials and methods

2.1 Broodstock husbandry and sperm collection

Semen used in the present study was collected from broodstock artificially reproduced from wild broodfish (please see Policar et al., 2007 for details). The fertilized

eggs were transported to the hatchery where hatched larvae were reared in identical controlled conditions and fed to maturity age. The fish were maintained at a density of 100 fish (mean total length and body weight ± S.E.M: 183 ± 20 mm and 50 ± 17 g, respectively) per tank (700 L). Recirculating system water flow was maintained at 10 L min^{-1}. Water temperature, dissolved oxygen and pH were 21.5 ± 0.5 °C, 6.8 ± 0.3 mg O_2 L^{-1} and 6.8 ± 0.3, respectively. The light-dark regime was increased 1 h per month during the reproductive season (10L: 14D, 11L: 13D and 12L: 12D in February, March and April, respectively). The fish was fed commercial diet (Karpico™ 33% crude protein and 6% fat) containing frozen chironomid larvae (*Chironomus plumosus*). Total daily food rations were 1.5% of total body weight of fish. In March and April, sperm from 19 spermiating males were collected with a syringe (5 ml) fitted with a plastic needle. We attempted to collect all the semen at each stripping. Special care was taken to avoid semen contamination by urine, mucus or blood. The external urogenital pore was wiped dry with paper towel before stripping. Syringes of semen were immediately placed on crushed ice (0 – 1 ^{o}C) and transported to the laboratory for later analyses.

2.2. Sperm volume, concentration and total number of spermatozoa

Sperm volume and concentration were measured and expressed as ml and billions of sperm per ml, respectively. Determination of spermatozoa concentration was measured by counting, following the method of Alavi et al., (2007). Semen was diluted 2 times, each 100-fold with 0.7% NaCl (final dilution rate: 10000). A haemocytometer was used to determine spermatozoa concentration. A drop of diluted semen (10 µl) was placed onto a haemocytometer covered with a coverslip and left for 10 min to allow sperm sedimentation before 16 cells (0.1 mm depth and 0.2 length) were counted. Total number of spermatozoa is recounted as spermatozoa concentration × sperm volume.

2.3. Determination of ion composition and osmolality of seminal plasma

Semen samples were centrifuged first at 3000 rpm for 3 min, followed by a 10 min at 10 000 rpm and the supernatant was separated and stored frozen at -80^{o} C until analysis. The osmolality of seminal plasma was measured in duplicates per sample with a Vapour Pressure Osmometer (Wescor, USA). Chloride, potassium, and sodium ions were measured using Ion Selective Electrodes, ISE, (Bayer Healthcare, New York, USA) by indirect simultaneous measurement, which require dilution of the sample (Stocking et al., 1999). Calcium concentration was measured by flame photometry. Ionic composition was measured once per sample.

2.4. Effects of potassium, sodium and sucrose on sperm motility

To investigate the dynamics of sperm motility during activation, sperm of 10 males (with initial motility higher than 80 %) were directly diluted in an activation medium containing Tris-HCl 20 – 30 mM, buffered to pH 8.0 ± 0.2, (67 mOsmol Kg^{-1}) at ratio 1: 1000 – 1:2000 (sperm: activation medium).

To examine the effects of ions on sperm motility, K^{+} (KCl) (0, 50, 100 and 150 mM with osmolality of 3, 130, 215, 345 mOsmol kg^{-1}, respectively) and Na^{+} (NaCl) (0,

50, 100 and 150 mM with osmolality of 3, 125, 230, 350 mOsmol kg^{-1}, respectively) were used. To test whether spermatozoa become motile in a non-ionic solution, sucrose solutions at 0.0, 25, 50, 100, 200 and 300 mM (4, 30, 50, 95, 210, 330 mOsml kg^{-1}, respectively) were tested. Sperm of same three males were used to study the effects of ions and osmolality. All solutions were buffered with 20 mM Tris-HCl, adjusted to pH 8.0 ± 0.2. Distilled water was used as control (3 – 4 mOsmol kg^{-1}).

2.5. Observation and assessment of sperm motility

Sperm motility was triggered directly in activation medium at ratio 1: 1000 – 2000 and immediately recorded with a 3 CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (NIKON Optiphot 2, Japan). Five successive positions of the recorded sperm heads were measured from video frames using a video-recorder (SONY SVHS, SVO-9500 MDP, Japan) and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Analysis of sperm motility was carried out in triplicate for each sample. To avoid sticking of spermatozoa to the slide, 0.1 % BSA (v/v) was added.

2.6. Data analysis

Data presented are mean ± standard error of mean (S.E.). Before analysis of data by ANOVA, Kolmogrov–Smirnov's and Leven's tests were used for normality of data distribution and homogeneity of variances. ANOVA was used to find correlations among length and weight of males, sperm volume, spermatozoa concentration, total number of spermatozoa, ionic contents and osmolality of the seminal plasma and sperm motility (SPSS 10.0). Statistical comparisons were made with Duncan's test for the analysis of spermatozoa velocity and percentage of motile sperm (SPSS 10.0).

3. Results

Na^+, K^+, Ca^{2+} and Cl^- concentrations in seminal plasma were 74.3 ± 2.5, 85.6 ± 2.7, 0.4 ± 0.1, 125.0 ± 2.1 mM (n= 7), respectively. Osmolality of seminal plasma was 273.9 ± 2.7 mOsmol kg^{-1} (n= 19).

There were significant correlations between spermatozoa concentration – length of fish (R= +0.66, F= 6.3, $P < 0.05$, n= 10) and spermatozoa concentration – weight of fish (R= +0.80, F= 14.3, $P < 0.05$, n= 10). No significant correlations ($P > 0.05$) were observed between total number of spermatozoa – length of fish (R= +0.64, F= 4.9 , n= 10), total number of spermatozoa – weight of fish (R= +0.49, F= 2.3 , n= 10), sperm volume – length of fish (R= +0.30, F= 1.6 , n= 10) and sperm volume – weight of fish (R= +0.30, F= 1.6, n = 18).

Osmolality of the seminal plasma increased when the total number of spermatozoa (R= +0.66, F= 6.0, $P < 0.05$, n= 10) and sperm volume (R= +0.62, F= 7.6, $P < 0.01$, n= 17) were higher in males.

Sperm motility (R= +0.51, F= 13.0, $P < 0.01$, n= 17) and velocity (R= +0.46, F= 4.62, $P < 0.05$, n= 17) increased in samples with higher osmolality (Fig. 1). A negative correlation was found between sperm motility and K^+ (R= –0.51, F= 4.5, $P < 0.05$, n= 7).

There were positive correlations between sperm motility – Ca^{2+} (R= +0.84, F= 28.9, P < 0.001, n= 7), Na^{+} (R= +0.81, F= 23.8, P < 0.001, n= 7), and Cl^{-} (R= +0.84, F= 32.4, P < 0.001, n= 7).

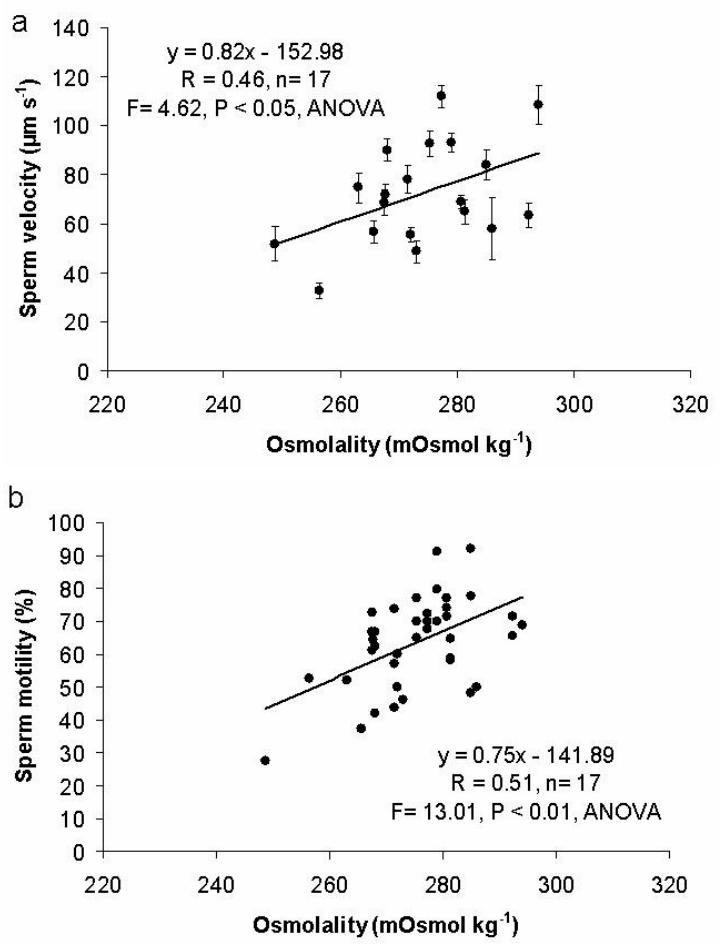

Fig. 1: Relationships between spermatozoa velocity (a µm s^{-1}) and sperm motility (b, %) with osmolality of the seminal plasma in *Barbus barbus*. The motility of spermatozoa was triggered in 20 – 30 mM Tris, pH 8.0 ± 0.2 at ratio 1: 1000 – 1:2000 (sperm: activation medium). Analysis of sperm motility was carried out in triplicate for each sample. Data presented are mean ± S.E.

A significant decrease in spermatozoa velocity and percentage of sperm motility was rapidly observed 30 s after triggering of motility of spermatozoa (Fig. 2, P < 0.001, n= 10). At 15 s post sperm activation, the percentage of sperm motility and spermatozoa velocity were 86.2 %, 100.3 µm s^{-1}, They decreased to 4.2 % and 7.1 µm s^{-1} at 90 s post sperm activation, respectively.

After triggering of sperm motility, beating waves propagated along the full length of flagella (Fig. 3). It is visible that the wave parameters change during sperm motility. The waves were completely absent at the end of the motility phase (90 s post sperm activation).

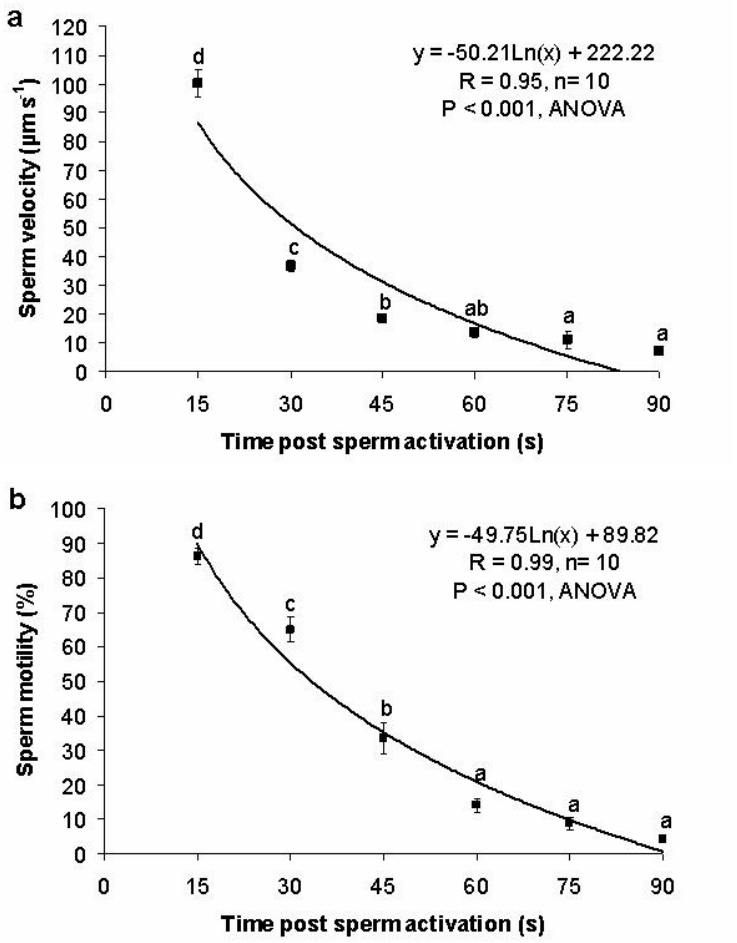

Fig. 2: Changes of spermatozoa velocity (a, µm s^{-1}) and percentage of motility (b %) *Barbus barbus*. The motility of spermatozoa was triggered in 20 – 30 mM Tris, pH 8.0 ± 0.2 at ratio 1: 1000 – 1: 2000 (sperm: activation medium). Analysis of sperm motility was carried out in triplicate for each sample. Data presented are mean ± S.E.

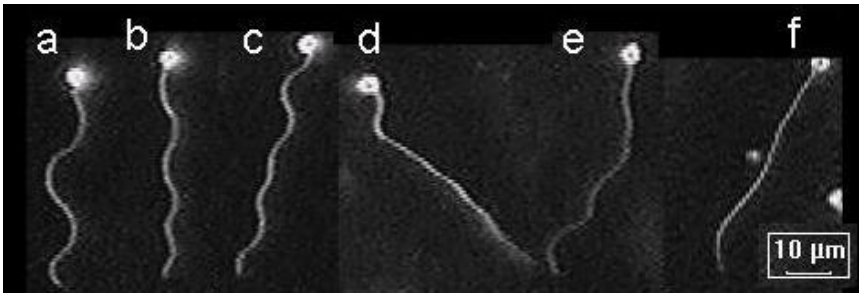

Fig. 3: Motility of *Barbus barbus* spermatozoon after activation in medium containing 100 mM NaCl, 20 mM Tris, pH 8.0 ± 0.2 at 10 (a), 15 (b), 35 (c), 45 (d), 60 (e) and 90 (f) s post sperm activation under a dark-field microscopy with stroboscopic flashing. Scale bar is 10 µm.

Osmolalities higher than 330 – 350 mOsmol kg^{-1} totally inhibited motility of sperm. Below that range, increasing the activating medium osmolality by NaCl, KCl or sucrose significantly increased the percentage of sperm motility (Fig. 4). In the presence of ions (NaCl or KCl), there was no significant difference in sperm motility between distilled water and 125 mOsmol kg^{-1} at 15 and 30 s post sperm activation. The lowest and highest percentage of sperm motility was observed in distilled water and also at 220 mOsmol kg^{-1} ($P < 0.05$). In sucrose solution, the highest percentage of sperm motility was observed from 50 to 215 mOsmol kg^{-1} at 15, 30, or 60 s post sperm activation (Fig. 4).

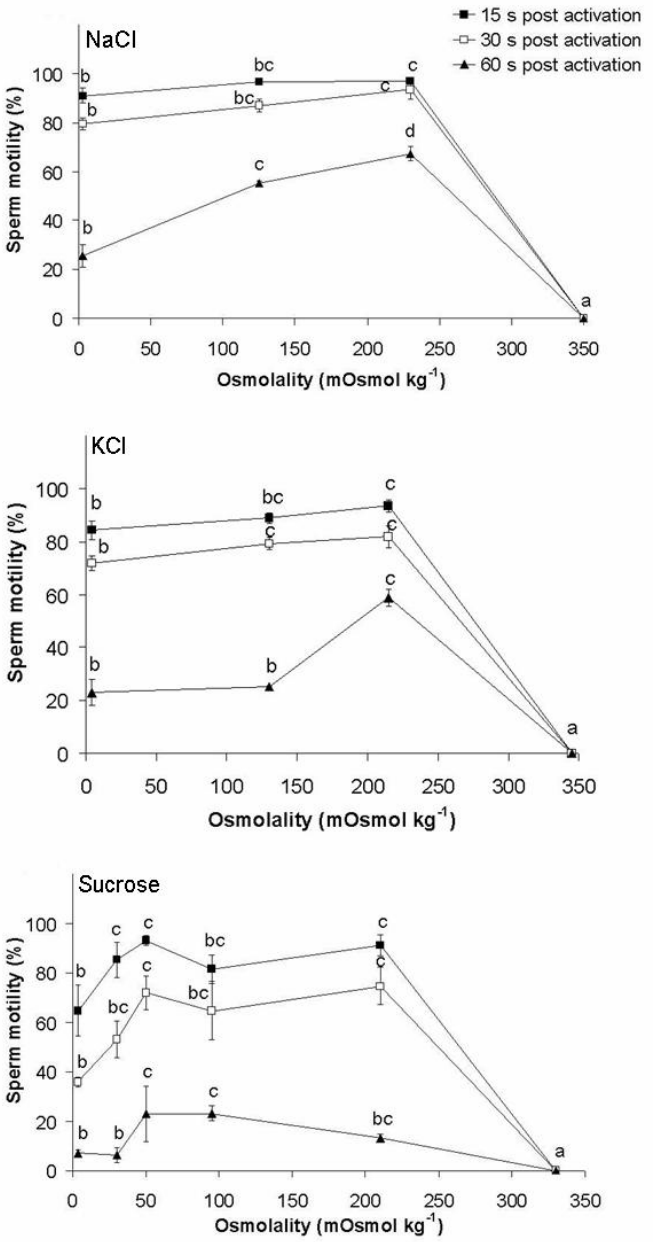

Fig. 4: Effects of osmolality on percentage of sperm motility (%) in *Barbus barbus* after activation in NaCl, KCl, or sucrose. At the same time post activation, values (mean ± S.E.) with the same letters are not significantly different (n= 3, $P > 0.05$). Analysis of sperm motility was carried out in triplicate for each male.

After activation of sperm in a solution containing NaCl, there was no significant differencein sperm velocity between distilled water (osmolality: 4 mOsmol kg^{-1}) and 125 mOsmol kg^{-1} ($P > 0.05$) (Fig. 5). In case of KCl, spermatozoa velocity was significantly lower in distilled water compared to those of 130 mOsmol kg^{-1} at 15 s post sperm activation but, did not differ significantly at 30 and 60 s post sperm activation (Fig. 5). After activation of sperm motility in sucrose, spermatozoa velocity increased significantly and was highest at 210 mOsmol k^{-1} at 15, 30 or 60 s post sperm activation ($P < 0.05$) (Fig. 5). The highest spermatozoa velocity was observed at 215 – 235 mOsmol kg^{-1} in an activating medium containing NaCl (128.6 µm s^{-1} at 15 s, 15.7 µm s^{-1} at 60 s), KCl (116.1 µm s^{-1} at 15 s and 13.1 µm s^{-1} at 60 s) or sucrose (137.2 µm s^{-1} at 15 s and 16.6 µm s^{-1} at 60 s) (Fig. 5).

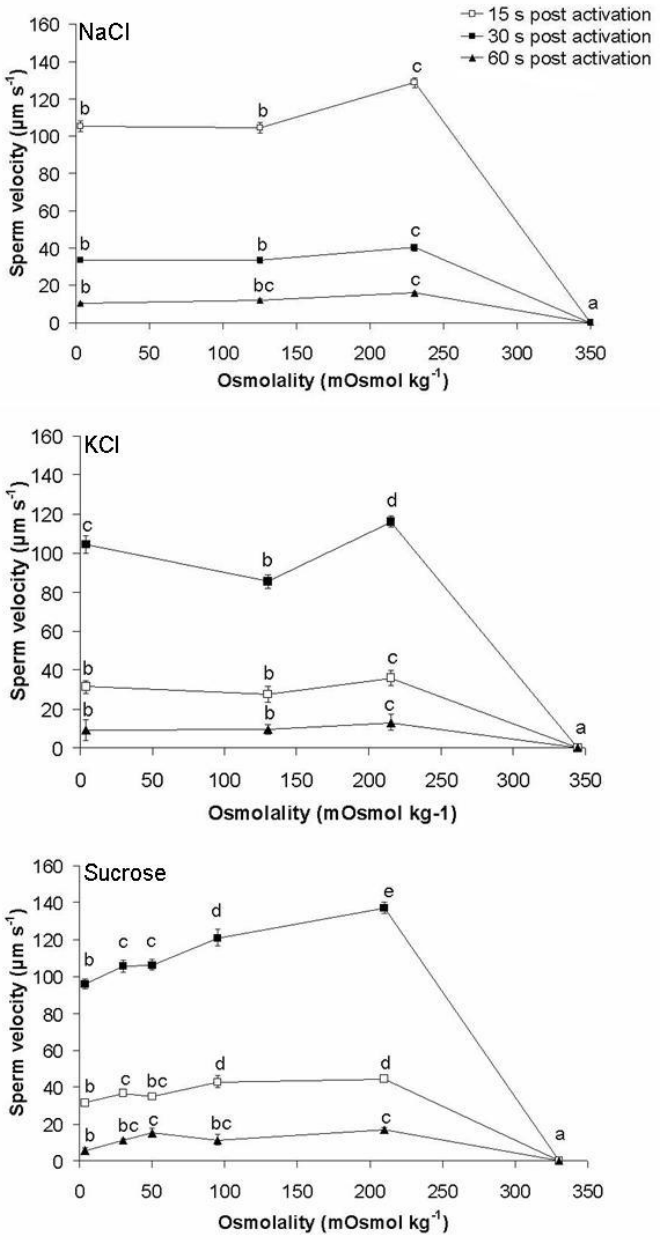

Fig. 5: Effects of osmolality on spermatozoa velocity (µm s^{-1}) in *Barbus barbus* after activation in NaCl, KCl, or sucrose. At the same time post activation, values (mean ± S.E.) with the same letters are not significantly different (n= 3, $P > 0.05$). Analysis of sperm motility was carried out in triplicate for each male.

Motility of spermatozoa was observed in a circular direction after activation of sperm in distilled water (a hypo-osmotic condition) (Fig. 6a), but trajectory of swimming sperm was observed in the straightforward direction by increasing osmolality of seminal plasma in NaCl (Fig 6b,c), KCl (6d) or sucrose (Fig. 6e).

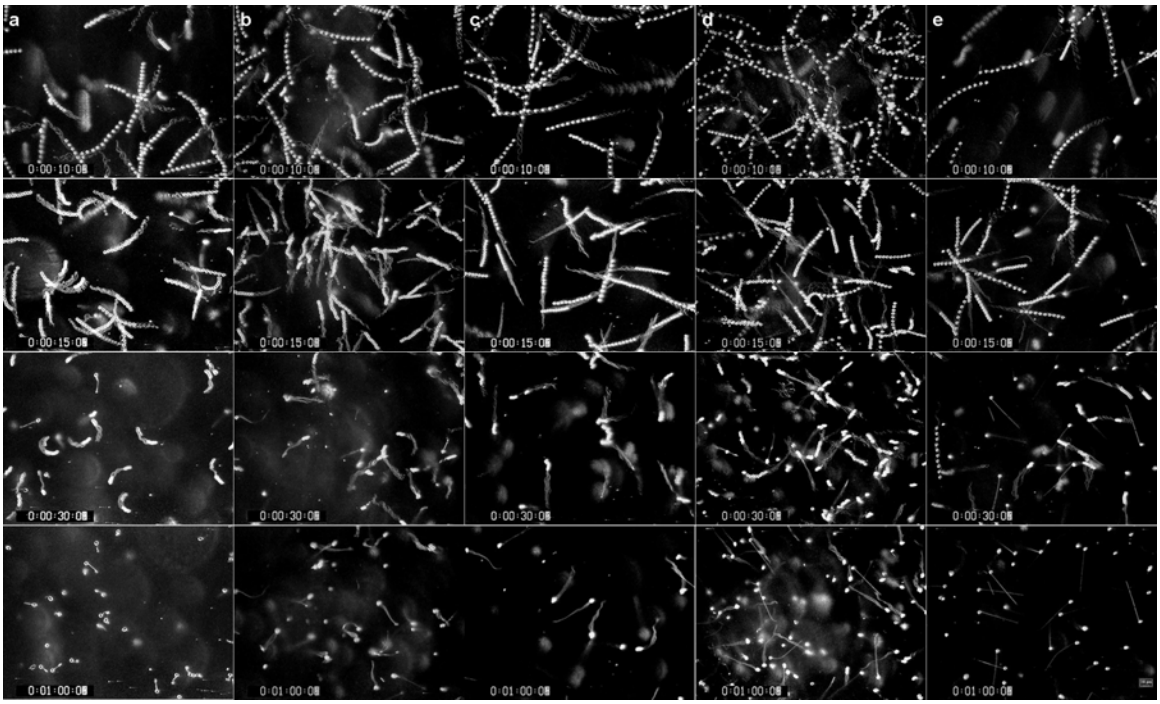

Fig. 6: Sperm head trajectories in *Barbus barbus* during motility after activation in (a) distilled water (a) and 50 mM NaCl (b), 100 mM NaCl (c), 100 mM KCl (d) and 100 mM Sucrose (e) + 20 mM Tris, pH 8.0 ± 0.2. Scale bar is 10 µm.

The tip of the flagellum of *B. barbus* spermatozoon became curled into a loop shape which shortened the flagellum after activation of sperm in distilled water (after 10 s post activation) or low osmolality of seminal plasma, less than 200 mOsmol kg^{-1} in NaCl, KCl, or sucrose, but not in high osmolality (Fig. 7). Such curling can be observed within 15 – 30 s post sperm activation in activating medium with osmolality 200 mOsmol kg^{-1}.

4. Discussion

Similar to other cyprinids, Na^+, K^+ and Cl^- are predominant ions in the seminal plasma of *B. barbus* (Table 1). Their concentrations are higher than Acipenseriformes (Alavi and Cosson, 2006; Alavi et al., 2004a). Depending on ionic concentrations, most of these ions are involved in regulating sperm motility either by contributing to the intracellular ionic composition or by regulating osmolality (Billard and Cosson, 1992; Morita et al., 2006). Osmolality of seminal plasma in cyprinids is higher than salmonids and acipenserids (Alavi and Cosson, 2006; Billard et al., 1995a,b). It may be due to higher concentrations of amino acids, peptides and proteins (Billard and Menezo, 1984; Lahnsteiner et al., 1994). Ionic contents as well osmolality differ among studies, which could be due to stripping time (early, middle or late spawning season), environmental conditions (which are stimulating regulatory functions of the endocrine system involved

in spermiation), frequency of stripping, and hormonal stimulation of spermiation (Billard et al., 1995a,b; Ciereszko, 2008). Variations in seminal plasma contents or osmolality can also result from contamination of sperm by urine (Perchec et al., 1995).

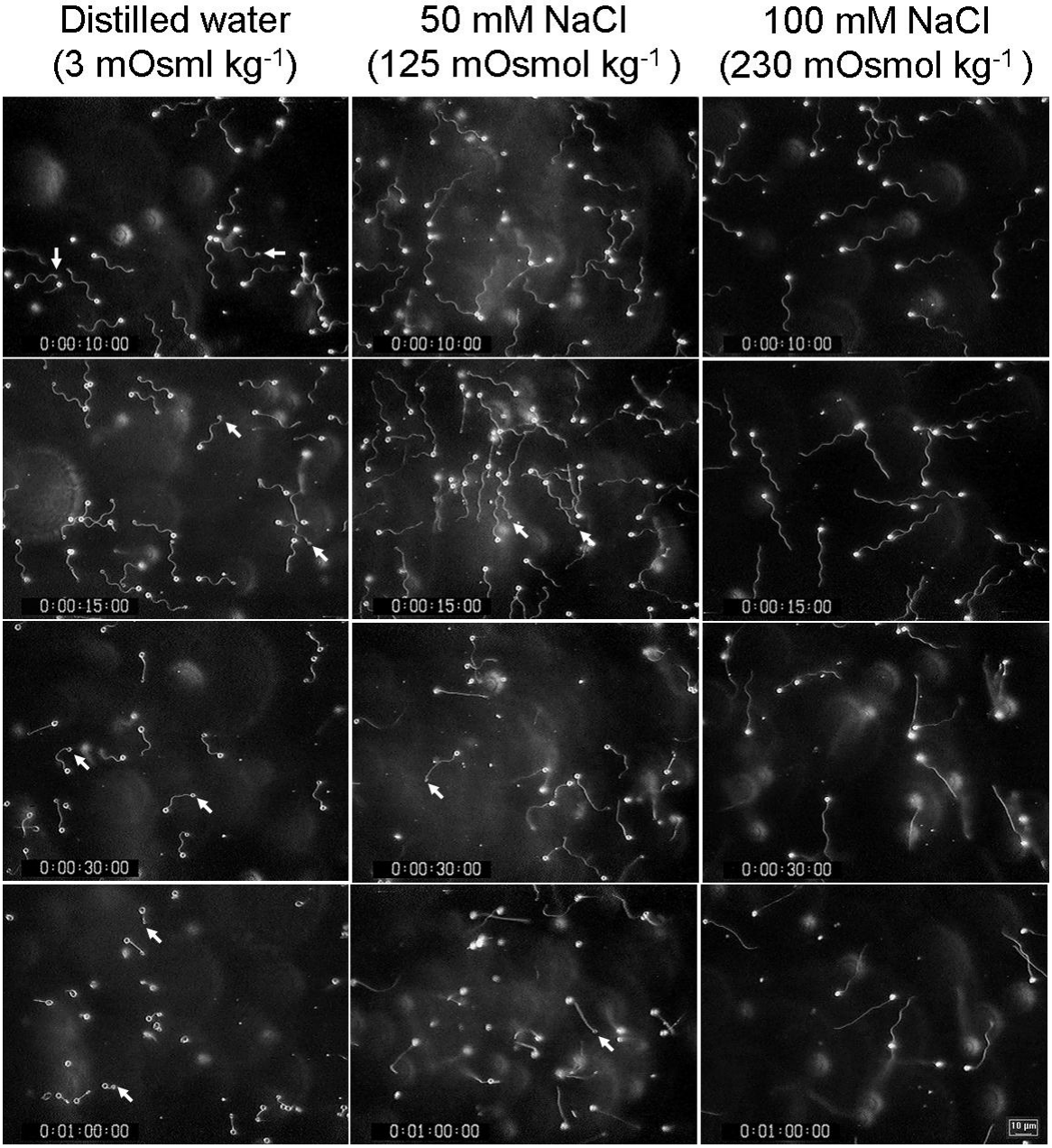

Fig. 7: Damage of flagella of spermatozoa after activation at different osmolality of activation medium in *Barbus barbus* under dark field microscopy with stroboscopic illumination at 10, 15, 30 and 60 s post activation of spermatozoa. The tip of the flagellum became curled into a loop shape which shortened the flagellum in distilled water or 50 mM NaCl (125 mOsmol kg^{-1}), but not at high osmolality (100 mM NaCl with 230 mOsmol kg^{-1}). NaCl solution was containing 20 mM Tris, pH 8.0 ± 0.2. Scale bar is 10 µm.

Table 1: Ionic (Na^+, K^+ and Ca^{2+}) composition and osmolality of seminal plasma in several species of cyprinids.

Species	Osmolality	K^+	Na^+	Ca^{2+}	Cl^-	Author(s)
Tinca tinca	230	1.93	18.41	0.6		Linhart et al., 2003
Cyprinus carpio	290 – 346	73 – 78	58 – 71	8 – 11*	96 – 111	Kruger et al., 1984
	258					Redondo-Muller et al., 1991
	302	82	75	2		Morisawa et al., 1983
		67.8	94	12.5		Clemens and Grant, 1965
	286	44	51	0.7		Plouidy and Billard, 1982
Ctenophoryngodon idella		35	81	1		Gosh, 1985
Alburnus alburnus	254 - 267	40	67			Lahnsteiner et al., 1996
Barbus barbus	268 – 276	84 – 85	70 – 76	0.3 – 0.4	121 – 125	Alavi et al., 2008
	249 – 294	75 – 98	59 – 78	0.1 – 0.6	113 – 128	Present study

*This value is in mg 100 mL^{-1}.

In this study, significant relationships were observed between weight and length of broodfish with total number of spermatozoa and spermatozoa concentration, but not with sperm volume. This could be related to endocrinological mechanism regulating hydration of sperm during spermiation (Nagahama, 1994; Billard et al., 1995b; Alavi and Cosson, 2006). Positive correlations between Na^+ and osmolality of seminal plasma with percentage of sperm motility have been reported in some freshwater teleosts. In contrast, negative correlations between K^+ and sperm motility have been reported (Billard and Cosson, 1992; Billard et al., 1995b; Lahnsteiner et al., 1996). Such relationships between ionic composition and sperm motility were not found in some species such as Chinook salmon, *O. tshawytscha* (Rosengrave et al., 2009) and Persian sturgeon, *Acipenser persicus* (Alavi et al., 2004a).

Spermatozoa velocity and percentage of motility in *B. barbus* showed a rapid decrease during the motility period as seen in Figure 3. Similar patterns of sperm motility parameters during activation have been shown in several freshwater teleost such as *Tinca tinca* (Linhart et al., 2003), *C. carpio* (Cosson et al., 1991; Billard and Cosson, 1992) and *Carassius gibelio* (Flajshans et al., 2008). It has been suggested that this rapid decrease in motility could be related to intracellular ATP content (Perchec et al., 1995; Billard et al., 1995b; Cosson, 2004; Burness et al., 2005).

Beating of the axoneme during sperm motility produces wave(s) along the flagellum. Quantitative wave parameters change as a factor of time post sperm activation

and vary among species. It was already shown that osmolality influences the wave parameters (Alavi et al., 2009). In *B. barbus*, it is clear that number of waves and curvatures in contrast to wave amplitude increase toward the end of swimming period of spermatozoa (Fig. 3). Ishijima et al., (1998) clarified that the number of waves on the flagellum increases as the total length of the flagellum increased. As the length of flagellum of B. barbus (54 μm) differ from that of *Tinca tinca* (25 μm), *Rutilis rutilus* (36 μm), *Chondrostoma toxostoma* (42 μm), *Carassius auratus* (58 μm) (Baccetti *et al.,* 1984; Psenicka *et al.,* 2006; Alavi et al., 2008a), further studies are needed to find the possible correlations between quantitative wave parameters, flagellar length, spermatozoa velocity and ATP content of sperm.

Several studies showed that environmental factors such as ions and osmolality stimulate the initiation of sperm motility by changing in the properties of the plasma membrane (including its osmotic potential and its ionic conductance; Morisawa, 1985; Morisawa et al., 1999; Krasznai et al., 2000). The present study provides the first report about *B. barbus* sperm response to ionic and osmolality effects. Generally, the highest percentage of sperm motility and velocity was observed at 200 mosmol kg^{-1}. Similar to almost all freshwater teleosts, the present study confirms that hyper osmolality compared to that of seminal plasma prevents sperm motility in the barbel. In salmonids and acipenserids, high K^+ concentrations in the seminal plasma play a main role for inhibiting the sperm motility (Ingermann, 2006; Alavi et al., 2004b).

Flagella of *B. barbus* spermatozoa after activation in hypo-osmotic condition showed damage within a few seconds post sperm activation. What we primarily noticed was that when the tip of the flagellum became curled into a loop shape. This loop shape resulted in a shortening of the flagellum and a restriction of wave development to the proximal part of the flagellum. This phenomenon has been already shown in *C. carpio* (Perchec et al., 1995; Linhart et al., 2008) and Northern pike, *Esox lucius* (Alavi et al., 2009). This observation may help to answer why the spermatozoa velocity is lower in distilled water compared to higher osmolalities close to the end of motility (Alavi et al., 2009). ATP content at the end of motility is also very low after sperm activation in distilled water compared to that of higher osmolality (Billard et al., 1995b).

Osmolality of activation medium is a key factor affecting sperm motility parameters (Cosson, 2004; Alavi et al., 2004b; Morita et al., 2006). However, the optimal osmolality associated with percentages of sperm motility or spermatozoa velocity varies among species. For example, the highest sperm motility parameters have been reported between 90 – 110 mOsmol kg^{-1} in common carp (Cosson et al., 1991; Billard et al., 1995b), 100 – 300 mOsmol kg^{-1} in tilapia (Linhart et al., 1999; Legendre et al., 2008), 100 – 150 mOsmol kg^{-1} in Eurasian perch, *Perca fluviatilis* (Alavi et al., 2007) and 125 – 235 in Northern pike (Alavi et al., 2009). The differences can be related to ionic composition and osmolality of the seminal plasma, time of stripping during reproductive season and environmental factors stimulating spermiation such as temperature and photoperiod (Suquet et al., 1994; Billard et al., 1995a; Alavi and Cosson, 2006; Alavi et al., 2008b). In common barbel, we suggest that K^+ ions play a key role in the activation sperm such as, *C. carpio* (Krasznai et al., 1995; and Java carp, *Puntius javanicus* (Morita et al., 2006). It has been confirmed that the motility of common carp sperm is highly decreased or suppressed in presence of K^+ channel inhibitors (Krasznai et al., 1995). Morisawa et al., (1983) shown that K^+ increases sperm motility and velocity in some

cyprinids. Krasznai et al., (2000) showed that the initiation of sperm motility has been reversibly inhibited in presence of Ca^{2+} channel blockers, suggesting that Ca^{2+} influx is the prerequisite for the initiation of carp sperm motility. Spermatozoa velocity in *B. barbus* reached about 120 – 140 μm s^{-1}, similar to those of *T. tinca* (Linhart et al., 2003) and *C. carpio* (Billard et al., 1995b), but perhaps slightly higher than *Carassius auratus* (120 μm s^{-1}, Morisawa et al., 1983). Spermatozoa velocity of course also depends on several other parameters such as head dimension (diameter of head), beat frequency, length of flagellum, and physical parameters of wave propagation (i.e., wave length and amplitude (Cosson, 2008; Cosson et al., 2008; Alavi et al., 2009). However, attention should be paid to changes of morphological parameters of sperm (flagellar length) and spermatozoa velocity during reproductive season (Christ et al., 1996; Alavi et al., 2008b).

In the present study, physiological relationships among length and weight of males, spermatozoa concentration, osmolality and ionic contents of seminal plasma and motility of spermatozoa were found that can be used as indictors for determining the quality of sperm. Osmolality of seminal plasma inhibits sperm motility in the seminal plasma, plays a major role for the initiation of sperm motility (Morisawa et al., 1983, 1999) and affects sperm motility parameters (Alavi and Cosson, 2006). As the same as most freshwater teleost, sperm motility last for a short period and its parameters (percentage of motility and velocity of spermatozoa) decrease rapidly during motility period that can be related to ATP contents of spermatozoa (Cosson, 2004). However, the tip of the flagellum became curled into a loop shape which shortened the flagellum after activation of sperm in hypo-osmotic conditions. The interactions between ions and osmolality remains to be studied in further studies as well flagellar wave pattern which can be specific in this species due to different flagellar length (Alavi et al., 2008a,b, 2009).

Acknowledgements

This work was supported by USB RIFCH 6007665809, GACR 523/09/1793 and NAZV QH91310 and QH71305. We are grateful to Ian A.E. Butts for commenting on the manuscript.

References

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Cosson, J., Karami, M., Abdoulhay, H., Mojazi Amiri, B., 2004a. Chemical composition and osmolality of seminal fluid of *Acipenser persicus*; their physiological relationship with sperm motility. Aqua. Res. 35, 1238–1243.

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A. 2004b. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction 128, 819–828.

Alavi, S.M.H., Rodina, M., Policar, T., Kozak, P., Psenicka, M., Linhart, O., 2007. Semen of *Perca fluviatilis* L: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology 68, 276–283.

Alavi, S.M.H., Psenicka, M., Policar, T., Linhart, O., 2008a. Morphology and fine structure of *Barbus barbus* (Teleostei: Cyprinidae) spermatozoa. J. Appl. Ichthyol. 24: 378–381.

Alavi, S.M.H., Psenicka, M., Rodina, M., Policar, T., Linhart, O., 2008b. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Teleostei: Cyprinidae) during the reproductive season. Aquat. Living Resour. 21, 75–80.

Alavi, S.M.H., Rodina, M., Viveiros, A.T.M., Cosson, J., Gela, D., Boryshpolets, S., Linhart, O., 2009. Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius* L.). Theriogenology *In press.*

Baccetti, B., Burrini, A. G., Callaini, G., Gibertini, G., Mazzini, M. and Zerunian, S., 1984. Fish germinal cells. I. Comparative spermatology of seven cyprinid species. Gamete Res. 10, 373–396.

Billard, R., Cosson, M.P., 1992. Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122–131.

Billard, R., Menezo, Y., 1984. The amino acid composition of rainbow trout (*Salmo gairdneri*) seminal fluid and blood plasma: a comparison with carp (*Cyprinus carpio*). Aquaculture 41, 255–258.

Billard, R., Cosson, J., Crim, L.W., Suquet, M., 1995a. Sperm physiology and quality. In: *Brood stock management and egg and larval quality*, Bromage, N.R., Roberts, R.J., (Eds.). Pp. 25–52. Blackwell Science. Amsterdam.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995b. Biology of sperm and artificial reproduction in carp. Aquaculture 124, 95–112.

Burness, G., Moyes, C.D., Montgomerie, R., 2005. Motility, ATP level and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochirus*). Comp. Biochem. Physiol. A 140, 11–17.

Christ, S., Toth, G.P., McCarthy, H.W., Torsella J.A., Smith, M.K., 1996. Monthly variation in sperm motility in common carp (*Cyprinus carpio*), assessed using computer-assisted sperm analysis (CASA). J. Fish Biol. 48, 1210–1222.

Ciereszko A., 2008. Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity and cryopreservation success in fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, R., Rafiee, G. (Eds.). Pp. 215–240. Alpha Science Ltd, Oxford.

Clemens, H.P., Grant, F. B., 1965. The seminal thinning response of carp, *Cyprinus carpio*, and rainbow trout, *Salmo gairdneri*, after injection of pituitary extract. Copeia 2, 174–177.

Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aqua. Int. 12, 69–85.

Cosson, J., 2008. The Motility apparatus of Fish Spermatozoa. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, R., Rafiee, G. (Eds.). Pp. 281–316. Alpha Science Ltd, Oxford.

Cosson, J., Billard, R., Cibert, C., Dreanno, C., Suquet, M., 1999. Ionic factors regulating the motility of fish sperm. In: *The male gamete: From basic to clinical applications,* Gagnon, C., (ed.). Pp. 161–186. Cache Rive Press, Vienna, IL.

Cosson, J., Billard, R., Redondo-Muller, C. Cosson, M.P., 1991. *In vitro* incubation and maturation of carp (*Cyprinus carpio*) spermatozoa. Bull. Inst. Zool., Acad. Sinica Monog. 16, 249–261.

Cosson, J., Groison, A.-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460–486.

Flajšhans, M., Rodina, M., Halačka, K., Vetešník, L., Gela, D., Lusková, V., Lusk, S., 2008. Characteristics of sperm of polyploid Prussian carp, *Carassius gibelio* (Bloch). J. Fish Biol. 73, 323–328.

Gosh, R.I., 1985. Energeticeskij obmen polovych kletok I embrionoy u ryb. Naukova dumka, Kiev. p. 147 (In Russian).

Hwang, P.C., Idler, D.R., 1969. A study of major cations, osmotic pressure, and pH in seminal components of Atlantic salmon. J. Fish. Res. Board Can. 26, 413–419.

Ingermann, R.L., 2006. Do sperm contribute to the buffering capacity of steelhead trout (Oncorhynchus mykiss) semen? Comp. Biochem. Physiol. A 144, 410–415.

Ishijima, S., Hara, M., Okiyama, M., 1998. Comparative studies on spermatozoan motility of Japanese fishes. Bull Ocean Res Inst Univ Tokyo 33, 139–152.

Krasznai, Z., Marian, T., Balkay, L., Gasper, R.Jr., Tron, L., 1995. Potassium channels requlate hypo-osmotic shock-induced motility of Common Carp (*Cyprinus carpio*) sperm. Aquaculture 129, 123–128.

Krasznai, Z., Marian, T., Izumi, H., Damjanovich, S., Balkay, L., Tron, L., Morisawa, M., 2000. Membrane hyperpolarization removes inactivation of Ca^{2+} channels leading to Ca^{2+} influx and initiation of sperm motility in the common carp. Proc. Nat. Acad. Sci. 97, 2052–2067.

Kruger, J.C., Smith, G.L., Van Vuren, J.H.J., Ferreira, J.T., 1984. Some chemical and physical characterestics of the semen of *Cyprinus carpio* and *Oreochromis mossambicus*. J. Fish Biol. 24: 263–272.

Lahnsteiner, F., Patzner, R.A., Weismann, T., 1994. The testicular main duct and the spermatic duct in some cyprinid fishes. II. Composition of seminal fluid. J. Fish Biol. 44, 459–467.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1996. Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. Fish Physiol. Biochem. 15, 167–179.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1998. Determination of semen quality of the rainbow trout by sperm motility, seminal plasma parameters and spermatozoal metabolism. Aquaculture 163, 163–181.

Legendre, M., Cosson, J., Alavi, S.M.H., Linhart, O., 2008. Sperm motility activation in the euryhaline tilapia *Sarotherodon melanotheron heudelotii* (Dumeril, 1859) acclimatized to fresh, sea or hypersaline waters. Cybium 32(2), 181–182.

Linhart, O., Walford, J., Sivaloganathan, B. Lam, T.J., 1999. Effects of osmolality and ions on the motility of stripped and testicular of freshwater- and seawater-acclimated tilapia, *Oreochromis mossambicus*. J. Fish Biol. 55, 1344–1358.

Linhart, O., Rodina, M., Bastl, J., Cosson, J., 2003. Urinary bladder, ionic composition of seminal fluid and urine with charcterization of sperm motility in tench (*Tinca tinca*). J. Appl. Ichthyol. 19, 177–181.

Linhart, O., Alavi, S.M.H., Rodina, M., Gela, D. and Cosson, J., 2008. Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (*Cyprinus carpio*). J. Appl. Ichthyol. 24, 386–396.

Lusk, S., 1996. Development and status of populations of *Barbus barbus* in the waters of the Czech Republic. Folia Zool. 45, (Suppl. 1), 39–46.

Morita, M., Okuno, M., Susilo, E.S., Setyo, B.P., Martarini, D., Harnadi, L., Takemura, A., 2006. Changes in sperm motility in response to osmolality/Ca^{2+} in three Indonesian fresh water teleosts: Goby (*Oxyeleotris marmorata*), Java carp (*Puntius javanicus*), and catfish (*Clarias batrachus*). Comp. Biochem. Physiol. A 143, 361–367.

Morisawa, M., 1985. Initiation mechanism of sperm motility at spawning in teleosts. Zool. Sci. 2, 605–615.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., Yasuda, K., 1983. Effect of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. J. Exp. Biol. 107, 95–103.

Morisawa, M., Oda, S., Yoshida M., Takai, H., 1999. Transmembrane signal transduction for the regulation of sperm motility in fishes and ascidians. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 149–160. Cache Rive Press, Vienna, IL.

Nagahama, Y., 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217–229.

Perchec, G., Jeulin, C., Cosson, J., Andre, F., Billard, R., 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747–753.

Plouidy, M.G., Billard, R., 1982. The chemical composition fluids of the gametes in the common carp, *Cyprinus carpio*,. In: *Reproductive Biology of Fishes*, Richter, C.J.J., Goos, H.J.Th. (Eds.). p. 134. PUDOC, Wagenington.

Policar, T., Kozak, P., Hamackova, J., Lepicova, A., Musil, J., Kouril, J., 2007. Effects of short-time *Artemia* spp. feeding in larvae and different rearing environments in juveniles of common barbel (*Barbus barbus*) on their growth and survival under intensive controlled conditions. Aquat. Living Resour. 20, 175–183.

Poncin, P., 1989. Effects of different photoperiods on the reproduction of the barbel, *Barbus barbus* (L.) reared at constant temperature. J. Fish Biol. 35, 395–400.

Psenicka, M., Rodina, M., Nebesarova, J., Linhart, O. 2006. Ultrastructure of spermatozoa of tench (*Tinca tinca*) observed by means of scanning and transmission electron microscopy. Theriogenology 66, 1355–1363.

Redondo-Muller, C., Cosson, M.P., Cosson, J., Billard, R., 1991. *In vitro* maturation of the potential for movement of carp spermatozoa. Mol. Reprod. Develop. 29, 259–270.

Rosengrave, P., Taylor, H., Montgomerie, R., Metcalf, V., McBride, K., Gemmell, N.J., 2009. Chemical composition of seminal and ovarian fluids of Chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. Com. Biochem. Physiol A. 152, 123–129.

Slechtova, V., Slechta, V., Luskova, V., Lusk, S., Berrebi, P., 1998. Genetic variability of common barbel, *Barbus barbus*, populations in the Czech Republic. Folia Zool. 47 (Suppl. 1), 7–20.

Stocking, C.J., Slater, J.M., Unwin, R., Walter, S., Folkerd, E., 1999. An automated technique for the simultaneous determination of cations in nanoliter volumes. Kidney Int. 56, 338–343.

Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnir, C., Fauvel, C., 1994. Sperm features in turbot (*Scophthalmus maximus*): A comparison with other freshwater and marine fish species. Aquat. Living Resour. 7, 283–294.

Chapter 3

Biology of sperm in *Perca fluviatilis* (Teleostei: Percidae)

Article 4: **Alavi, S.M.H.,** Rodina, M., Policar, T., Kozak, P., Psenicka, M., Linhart, O. 2007. Semen of *Perca fluviatilis* L: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology, 68: 276–283.

Article 5: **Alavi, S.M.H.,** Rodina, M., Hatef, A., Stejskal, V., Policar, T., Hamáčková, J., Linhart, O., Sperm motility and monthly variations of semen characteristics in *Perca fluviatilis* (Teleostei: Percidae). Czech Journal of Animal Science, Submitted.

Semen of *Perca fluviatilis* L: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility

S. M. Hadi Alavi, Marek Rodina, Tomáš Policar, Pavel Kozak, Martin Pšenička, Otomar Linhart

University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany 389 25, Czech Republic

Abstract

The objectives of the present study were to characterize sperm volume and density, seminal plasma indices (ionic contents and osmolality) and to study the effects of dilution ratio, ions and osmolality on sperm motility parameters (percentage of motile sperm and sperm velocity) in farmed European perch (*Perca fluviatilis* L.). The means of sperm volume (ml), sperm density ($\times 10^9$ sperm ml^{-1}) and total number of sperm (volume × density) per fish were 2.75 ± 0.51, 29.19 ± 3.15 and 82.19 ± 15.26. The seminal plasma osmolality (mOsm kg^{-1}), sodium, chloride, potassium and calcium ions concentrations (mM) were measured 298.07 ± 5.09, 130.97 ± 2.19, 106.75±2.37, 10.70 ± 0.61 and 2.41 ± 0.09 respectively. At 15 sec post activation of stripped sperm, the percentage of motile sperm (%) and sperm velocity (μm s^{-1}) were 91.90 ± 1.27 and 115.54 ± 1.25, respectively and decreased significantly following sperm activation ($P < 0.05$). The optimal sperm motility was observed when the sperm was prediluted in immobilizing solution (IS) at a ratio 1:50. Prediluted sperm showed the maximum velocity when activated in 2.5 mM Ca^{2+}, 50 mM K^+ and sucrose with osmolality 100 mOsm kg^{-1}. Neither Ca^{2+} nor K^+ showed a significant effect on the percentage of motile sperm at 15 sec post activation. Osmolality higher than 200 mOsm kg^{-1} significantly decreased the percentage of motile sperm, while osmolality of 300 mOsm kg^{-1} or above totally suppressed sperm motility.

Key words: osmolality, dilution ratio, Calcium, Potassium, sperm motility

1. Introduction

Basic knowledge of seminal plasma composition, sperm production indices (sperm volume and density), behaviour of sperm relative to environmental conditions such as ions and osmolality can expand understanding of intra- and inter-species variations in fish (Alavi and Cosson, 2005, 2006).

Sperm of both freshwater and seawater fish are immotile in the testis but acquire the potential for motility during transfer from the testis to the sperm duct (Morisawa and Morisawa, 1986). The inhibition of sperm motility in most species is due to the seminal plasma osmolality (Billard et al., 1995), but a high concentration of potassium ions plays a major inhibitory role in Salmonidae (Billard and Cosson, 1992) and Acipenseridae (Cosson and Linhart, 1996; Cosson et al., 1999; Alavi et al., 2004). In general, motility of sperm is induced by hypo-osmotic pressure in freshwater fishes and by hyper-osmotic pressure in marine fishes.

Sperm motility is also influenced by several parameters, such as temperature, pH, ions (including Na^+, K^+, Ca^{2+}), osmolality and dilution ratio in either activation or immobilizing solutions (Billard et al., 1995; Cosson et al., 1999; Cosson, 2004; Alavi and Cosson, 2005, 2006). Understanding effects of these parameters are helpful in developing optimal methods of artificial reproduction and can provide information for

the development of better short and long term storage conditions for sperm (Billard et al., 1995). Little is known regarding sperm of European perch specially in terms of factors triggering and regulating motility of sperm exposed to extracellular ions (K^+, Ca^{2+}) and osmolality, as well as the role of the dilution ratio of sperm to an immobilizing solution (Lahnsteiner et al., 1995).

The objectives of the present study were (a) to characterize aspects of semen including sperm volume and density, seminal plasma parameters (ionic contents and osmolality), (b) to determine the main triggers for sperm activation, (c) to study the effects of dilution ratio in an immobilizing solution, and K^+ ions and osmolality on sperm motility in farmed *Perca fluviatilis* L.

2. Materials and methods

2.1. Semen collection

Juveniles from a semi-artificial reproduction of natural broodfish in pond were captured and transported to the hatchery and reared in natural condition (pond) until maturation. Semen samples were collected from 16 farmed three year-old *P. fluviatilis* (Table 1) with a syringe while pressure was applied to the fish's abdomen. Care was taken to collect all the available semen and to avoid contamination by urine. The samples were kept on ice during the investigation. Experiments were carried out immediately after transport of semen to the Laboratory of Sperm Physiology and Biochemistry, Research Institute of Fish Culture and Hydrobiology.

2.2. Determination of sperm volume and density

Sperm volume (ml) and sperm density (billions of sperm per ml) were measured according to methods previously described (Caille et al., 2006). Semen was diluted 1000 x in physiological saline, a droplet was placed on a Burker cell haemocytometer (depth 0.1 mm), left for 10 min to allow sedimentation, then 16 cells of haemocytometer were counted at 200 x magnification. Weight and total length of fishes were measured to estimate their possible correlations with sperm volume and density.

2.3. Determination of seminal plasma indices

Semen samples were centrifuged first at 3000 rpm for 3 min, followed by a 10 min at 10 000 rpm and the supernatant was stored as frozen at -80^o C for analysis of ionic composition and osmolality. Osmolality was measured with an osmometer (Model 2020, Advanced Instruments, Norwood Massachusetts, USA) by the freezing-point method. Chloride, K^+, and Na^+ were measured using Ion Selective Electrodes, ISE, (Bayer HealthCare, New York, USA) by indirect simultaneous measurement at the Medicine Laboratory, Central hospital in Ceske Budejovic (Czech Republic). Calcium concentration was measured by flame photometry.

2.4. Evaluation of sperm motility and determination of optimum dilution ratio

Sperm motility was recorded after activation using a 3 CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (NIKON Optiphot 2, Japan) at frequency set to 50 Hz. The successive positions of the recorded sperm

heads were measured from video frames using a video-recorder (SONY SVHS, SVO-9500 MDP, Japan) and computed from five successive frames by a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Sperm motility parameters including velocity (the mean of velocity of motile sperm cells) and percentage of motile sperm were measured.

A two step method was used to evaluate sperm motility in all experiments (Billard and Cosson, 1992). Semen was diluted in an immobilizing solution (IS) containing 200 mM NaCl and 2.38 mM $NaHCO_3$, pH 7.5 (osmolality 380 mOsm kg^{-1}) at three different dilution ratios (1: 25, 1: 50 and 1: 100) to determine the optimal predilution level. Temperature of IS was 2-4 ^{o}C. Then, prediluted sperm were activated by dilution in an activation solution (AS) containing 20 mM Tris-Cl at pH 8.0 at a ratio 1:50 (1 µl prediluted sperm: 49 µl AS). The optimal dilution ratio at first step was 1:50 and subsequent experiments were carried out at this ratio. To avoid sperm adhesion to the glass slide, 0.1 % bovine serum albumin (BSA) was added to AS. In order to prevent the possible ATP and motility regeneration which have been observed in common carp (Perchec et al., 1995), undiluted sperm was prediluted in IS and within less than 30 sec, motility was activated in AS in all experiments.

Table I. Descriptive statistics of sampled sperm, volume, density, seminal plasma characteristics and sperm motility parameters (sperm velocity and percentage of motile sperm) in the perch, *Perca fluviatilis*. Standard error of mean (S.E.) and standard deviation (S.D.). In case of percentage of motile sperm and their velocity, data with different letters are significant ($P < 0.05$).

Parameter	N	Min.	Max.	Mean	S.E.
Length and weight of sampled fish					
Fish length (mm)	16	198.00	214.00	206.44	1.16
Fish weight (g)	16	88.47	117.34	104.21	2.05
Sperm production indices					
Sperm volume (ml)	16	0.55	6.78	2.75	0.51
Sperm density ($\times 10^9$ spz ml^{-1})	16	3.28	43.91	29.19	3.15
Total number of spermatozoa	16	1.97	196.83	82.19	15.26
Seminal plasma characteristics					
Sodium (mM)	12	112.50	138.30	130.97	2.19
Chloride (mM)	12	88.00	115.00	106.75	2.37
Potassium (mM)	12	7.67	15.02	10.70	0.61
Calcium (mM)	12	1.69	2.81	2.41	0.09
Osmolality (mOsmol kg^{-1})	14	255.00	334.00	298.07	5.09
Percentage of motile sperm at time period post activation (%)					
15 sec	15	66.67	100.00	91.90^{a}	1.27
30 sec	15	8.86	67.65	35.49^{b}	2.17
45 sec	15	0.00	20.00	7.04^{c}	0.71
Sperm velocity at time period post activation (µm s^{-1})					
15 sec	15	14.74	214.06	115.54^{a}	1.25
30 sec	15	3.75	67.62	12.96^{b}	0.20
45 sec	15	0.00	21.16	6.68^{c}	0.20

2.5. Effects of potassium and calcium ions and osmotic pressure

The effects of K^+ (KCl) (5.0, 20.0 and 50.0 mM) and Ca^{2+} ($CaCl_2$) (0.5, 2.5 and 5.0 mM) on sperm motility were tested on four and three fish samples,

respectively. Sucrose solutions at 100, 200 and 300 mOsm kg^{-1} were used to test the effects of osmotic pressure on sperm motility in five fish samples using the two step method already described. Buffered distilled water containing 20 mM of Tris-HCl adjusted to pH 8.0±0.2 was used as a control in all experiments.

2.6. Data analysis

After controlling normality of data by Kolmogrov–Smirnov's test, ANOVA was used to find correlations among seminal plasma parameters (SPSS 10.0). A stepwise multi-regression model (Statgraphic Plus 2.2) was used to identify a possible regression model between all measured parameters and sperm motility (velocity and percentage of motile sperm). Statistical comparisons were made with Duncan's test for the analysis of sperm velocity and percentage of motile sperm (SPSS 10.0).

3. Results

3.1. Characterization of sperm indices

Sperm volume and density and total number of sperm per fish showed high variations among samples (Table 1). Significant polynomial correlations were observed between sperm volume and total length of fish (R^2= 0.43, P<0.05) and total number of sperm per fish and weigh of fish (R^2= 0.33, P<0.05). Sodium and chloride were predominant ions in the seminal plasma (Table 1). The mean value of osmolality for the seminal plasma was 298.07 ± 5.09 mOsm kg^{-1}. The percentage of motile sperm and sperm velocity decreased significantly soon after sperm activation (Table 1).

A stepwise multiple linear regression model showed a positive relationship between percentage of motile sperm (DV_{PMS}) as the dependent variable and mass of broodfish (V_2), and sperm density (V_4), Na^+ (V_5), K^+ (V_7), and a negative relationship between the percentage of motile sperm and sperm volume (V_3) and Cl^- (V_6). The fitted multiple regression model was:

$DV_{PMS} = 54.89 + 1.04V_2 – 3.64V_3 +0.40V_4 + 1.35V_5 – 2.46V_6 + 1.49V_7$ (ANOVA, R2= 0.87, Df: 6, F-ratio: 5.60, $P < 0.05$).

Other measured parameters (Ca^{2+} (V_8) and osmolality (V_9)) did not show significant correlation with the percentage of motile sperm.

3.2. Sperm motility

The highest and lowest sperm velocities were observed when the sperm was diluted in IS at ratios 1:100 and 1:50, respectively ($P < 0.05$) (Fig. 1a). No significant differences were observed in the percentage of motile sperm at 15 sec post activation ($P > 0.05$), whereas at 30 and 45 sec post activation, the maximum sperm velocity and the highest percentage of motile sperm were observed when the sperm was prediluted in IS at 1:50 ($P < 0.05$) (Fig. 1b). Subsequent experiments were therefore carried out at a dilution ratio of 1:50.

Increasing concentration of potassium ions in AS increased sperm velocity. The highest sperm velocity was observed when 50 mM K^+ was present in AS [Fig. 2(a)], but K^+ did not affect significantly the percentage of motile sperm (Fig. 2b). In general, the highest percentage of motile sperm was observed at K^+ concentrations above 20 mM when measured at 15 sec post activation (Fig. 2b).

Maximum sperm velocity was observed after dilution of sperm into a medium with osmolality of 100 mOsm kg^{-1} ($P < 0.05$) (Fig. 3a). The percentage of motile sperm did not differ significantly between control and AS at 100 mOsm kg^{-1} when measured at 15 sec post activation (Fig. 3b), but it was lowest after dilution of sperm into the medium at 200 mOsm kg^{-1}. At 30 sec post activation, sperm velocity was the same in AS at 100 compared to 200 mOsm kg^{-1}, but the percentage of motile sperm was significantly lower in AS at 200 mOsm kg^{-1}. The sperm motility of *P. fluviatilis* was inhibited in AS at 300 mOsm kg^{-1} (data not shown).

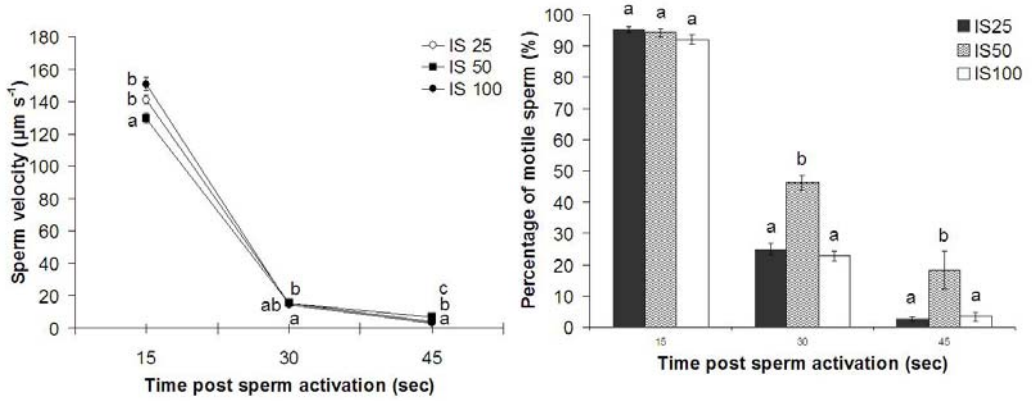

Fig. 1: Effect of dilution ratio in immobilizing solution on *Perca fluviatilis* stripped sperm velocity (a) and percentage of motile sperm (b). Values represent the mean ± S.E. of six male samples in triplicate. The velocity values are corresponding to motile sperm cells. Values with the same superscript are not significantly different at the same time post activation ($P > 0.05$, Duncan test).

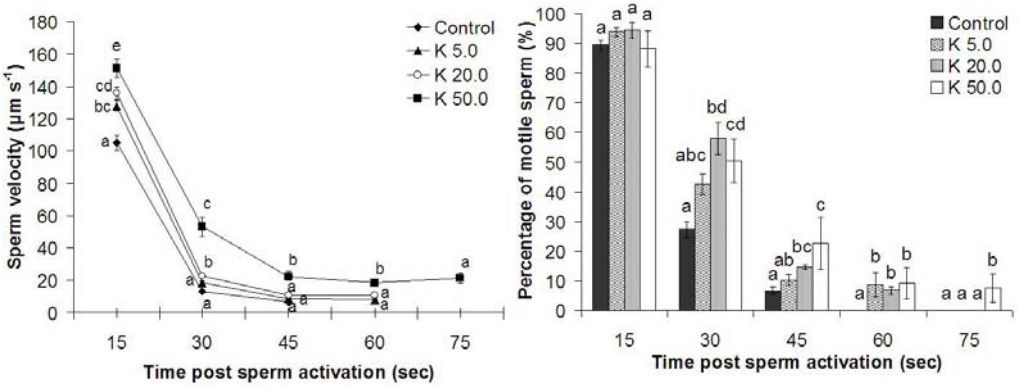

Fig. 2: Effect of potassium ion concentrations on *Perca fluviatilis* stripped sperm velocity (a) and percentage of motile sperm (b). Values represent the mean ± S.E. of four male samples in triplicate. The velocity values are corresponding to motile sperm cells. Values with the same superscript are not significantly different at the same time post activation ($P > 0.05$, Duncan test)

At 15 sec post activation, the highest sperm velocity occurred after activation of the sperm in AS containing 2.5 mM Ca^{2+} ($P < 0.05$) (Fig. 4a), but no significant difference was observed in the percentage of motile sperm ($P > 0.05$) (Fig. 4b). In general, these results show that 2.5 mM of Ca^{2+} has an optimum effect by increasing both sperm velocity and percentage of motile sperm.

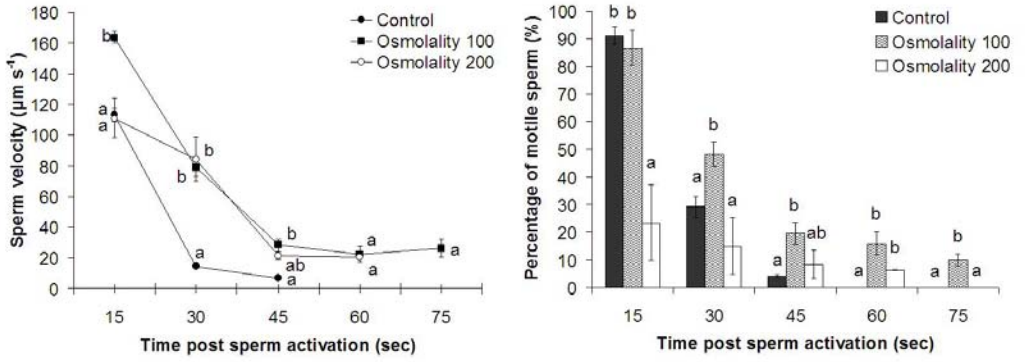

Fig. 3: Effect of osmolality on *Perca fluviatilis* stripped sperm velocity (a) and percentage of motile sperm (b). The velocity values are corresponding to motile sperm cells. Values represent the mean ± S.E. of five male samples in triplicate. Values with the same superscript are not significantly different at the same time post activation ($P > 0.05$, Duncan test)

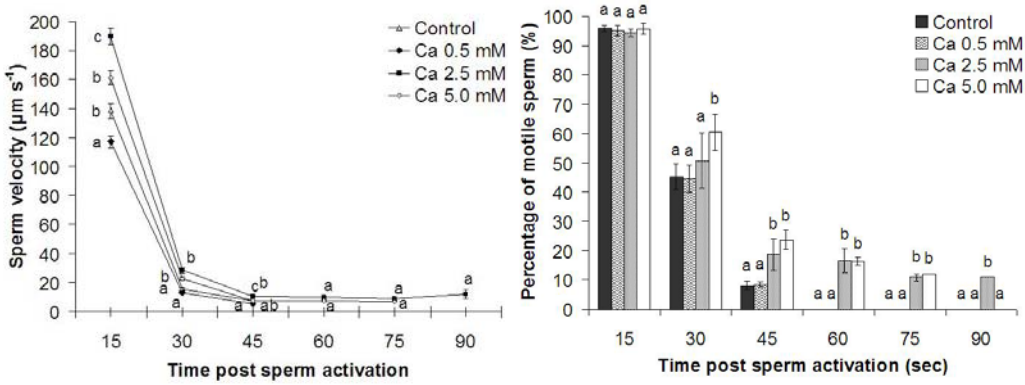

Fig. 4: Effect of calcium ions concentrations on *Perca fluviatilis* stripped sperm velocity (a) and percentage of motile sperm (b). The velocity values are corresponding to motile sperm cells. Values represent the mean ± S.E. of three male samples in triplicate. Values with the same superscript are not significantly different at the same time post activation ($P > 0.05$, Duncan test)

4. Discussion

4.1. Characterization of sperm indices

In perch, both positive (Wirtz and Steinmann, 2006) and negative (Kucharczyk et al., 1996) correlation between stripped sperm volume and body mass have been reported. In the present study, no significant correlation was observed. In this study, sperm density was similar to values obtained by Krol et al., (2006), higher than in the study carried out by Lahnsteiner et al. (1995) and lower than results reported by Wirtz and Steinmann, (2006). The observed differences in sperm density and volume support the assumption that sperm production in perch, depends on broodfish rearing conditions, methods of spawning induction, duration of broodstock participation in spawning, and duration and time of spermiation within the reproductive season (Alavi et al., 2008).

Nevertheless, mean sperm density in perch was higher than in many teleost fishes such as common carp and rainbow trout (Billard et al., 1995; Alavi et al., 2008) but the variation among males was considerably larger. Such variations were probably due to differences in the spawning behaviour of the species. The volumes of sperm in this study of perch were similar to those found by Piironen and Hyvarinen, (1983).

Studies of seminal plasma parameters are crucial for two reasons. First for understanding the basic biochemical processes occurring during the maturation of sperm in the male reproductive accessory organ (Miura and Miura, 2003), the spontaneous motility of sperm in the sperm duct (Cosson, 2004) and the initiation of sperm motility after release into the external environment (Cosson et al., 1999; Morisawa et al., 1999). Secondly, for evaluation of the inter- and intra-specific aspects of reproductive ability (Linhart et al., 1991; Billard et al., 1995; Alavi and Cosson, 2005, 2006; Alavi et al., 2008. Some of the observed differences between the present study and that of Lahnsteiner et al., (1995) were due to several parameters including the period of spermiation during the reproductive season (Billard and Coson, 1992; Koldras et al., 1996), the phagocytosis of sperm cells in the testis during degenerative processes (Billard and Takashima, 1983), endocrine parameters such as steroids during spermatogenesis and spermiation (Marshall et al., 1988) and the various strategies used to artificially induce spawning (Linhart et al., 2003), the duration and intensity of broodfish anaesthesia (Dietrich et al., 2005), stripping frequency and time of the spawning season (Buyukhatipoglu and Holtz, 1984; Alavi et al., 2006).

4.2. Sperm motility

The duration of perch sperm motility is very short, and sperm velocity significantly decreases with time post activation.

Dilution ratio of sperm is of interest in understanding its effect on fertilization success relative to sperm motility. Billard, (1983), Billard et al., (1995b) and Linhart et al. (2004) found that changes of dilution ratio of fresh or stored sperm with water or saline solutions changed the fertilizing ability of the sperm in teleost fishes such as common carp and trout (Billard et al., 1995a). Recently, it was shown that the dilution ratio of sperm in activation medium affects fertilizing ability through changes in sperm motility parameters such as sperm velocity, percentage of motile sperm and total period of sperm motility (Rodina et al., 2004). Since the duration of motility is short, and since sperm motility characteristics vary during the phase of motility, dilution becomes a key issue, because the volume of dilutent determines the dynamics of sperm activation (Billard and Cosson, 1992). Following a large dilution, a homogenous sperm suspension is obtained which is suitable for observing synchronous motility and for investigating the biochemical changes that occur during and after activation. A two-step dilution has been suggested, which consists of first diluting in an immobilisation solution (IS) and then inducing sperm activation in an activation solution (AS) (Billard et al., 1995a; Rodina et al., 2004). Several studies have revealed that the role of IS is not only to stop spontaneous motility of sperm due to possible contamination by urine (Linhart et al., 2003; Rodina et al., 2004) or by skin mucus (Kowalski et al., 2003), but also to allow the recovery of ATP (Cosson, 2004). However, the composition of IS depends on the seminal plasma composition and its main component preventing sperm motility. Further studies are needed to investigate effects of incubation of sperm in IS on ATP and motility regeneration.

High concentrations of K^+ significantly increased the percentage of motile sperm and the sperm velocity, but its concentration in the seminal plasma was not high enough to account for the inhibition of motility in semen. It means that the motility of perch sperm is inhibited by the high osmolality of the seminal plasma, and exposure to hypo-osmotic media with osmolality lower that of seminal plasma triggers sperm motility.

Taken together, these results indicated that osmolality isotonic to the seminal plasma suppresses sperm motility in perch and exposure to hypo-osmotic medium induces the initiation of sperm motility. The same controlling effects of K^+ have been reported in salmonids (Billard and Cosson, 1992; Morisawa et al., 1983) and sturgeons (Cosson and Linhart, 1996; Alavi et al., 2004). It has also been shown that sperm motility inhibition by high concentrations of K^+ ions varies during the spawning season, so that at the beginning and at the end of the spermiation period, a high percentage of sperm become motile even in the presence of a high K^+ concentration (40 and 80 mM) (Billard and Cosson, 1992). This suggests that alteration in the concentration of K^+ in the semen is accompanied by sperm sensitivity to K^+ level which is a species-specific characteristic (Cosson et al., 1999; Alavi and Cosson, 2006).

Recent research concerning the role of K^+ channels in sperm motility in carp has confirmed a reduction or suppression of sperm motility in the presence of K^+ channel inhibitors (Krasznai et al., 2000; Cosson, 2004). Therefore, K^+ channels may exert an effect on sperm motility and regulate hypo-osmotic shock in the case of common carp sperm (Morisawa et al., 1999), a feature which may be shared with perch sperm.

The present study reveals that perch sperm behaves similarly to that of other fresh water species (Billard et al., 1995a; Cosson, 2004; Alavi and Cosson, 2006; Alavi et al., 2008) in which the osmotic range of media for activation of sperm motility is hypotonic compared to seminal plasma osmolality. The present study confirms the results of Lahnsteiner et al., (1995) which reported the highest sperm motility of perch in a solution containing NaCl, glucose or sucrose with osmolality of 100 mOsm kg^{-1}. Between study differences observed in osmolality required to suppress sperm motility (350 mOsm kg^{-1}, Lahnsteiner *et al.*, 1995 *vs.* 300 mOsm kg^{-1}, present study) are probably related to the differences in seminal plasma composition and to the components that were used for preparation of media.

Several studies showed the role of millimolar concentrations of calcium in an increase in sperm motility parameters, including total period of activity, percentage of motile sperm and sperm velocity (Baynes et al., 1981; Cosson, 2004; Alavi and Cosson, 2006). The present study showed that Ca^{2+} concentration of 2.5 mM increased both the percentage of motile sperm and the sperm velocity in perch. However, the results in literature demonstrate the biosensivity of fish sperm to increases in Ca^{2+} concentration (Cosson et al., 1999; Alavi et al., 2004; Alavi and Cosson, 2006). The present study also showed a lower percentage of motile sperm and sperm velocity at Ca^{2+} concentration of 5.0 mM compared to 2.5 mM. The role of Ca^{2+} in sperm cell signalling has been studied in some teleost fishes. Preliminary results indicate that the concentration of intracellular Ca^{2+} induced initiation of motility (Christen et al., 1987; Boitano and Omoto, 1991; Cosson et al., 1999; Morisawa et al., 1999). Recent studies have shown that prevention of the entrance of Ca^{2+} ions into the cell by a voltage-dependent blocker of calcium channels results in the complete inhibition of motility in common carp (Krasznai et al., 2000). Also, the increase of intracellular free Ca^{2+} is produced by a flux of external Ca^{2+} into the cell

rather than by a mobilization of internal Ca^{2+} stores (Krasznai et al., 2000, 2003). In addition, internal Ca^{2+} concentration has been shown to affect linearity of sperm tracks, leading to circular motion at high Ca^{2+} concentration (Billard and Cosson, 1992; Cosson et al., 1999).

Results of the present study suggest that hypo-osmotic shock generates an osmotic gradient between the intracellular and extracellular medium to balance the osmolality on both sides of the plasma membrane through an influx of water as well increase in ion channels activities. The present study also suggests that potassium might be involved in the activation mechanism for perch sperm, similar to that described for common carp (Perchec-Poupard et al., 1998; Morisawa et al., 1999; Krasznai et al., 2000), through the changes of the plasma membrane potential, which was shown to be an effector of flagellar movement (Gatti et al., 1990). In addition to potassium, osmolality-dependent cell signalling system is the major parameter controlling perch sperm motility. Perch sperm motility is initiated in hypo-osmotic environmental conditions probably through depolarization. Nevertheless, further studies are needed to determine which factor triggers the intracellular events and whether it is a pH-dependent mechanism.

The present study of perch sperm showed high individual variations in term of sperm density and volume. High density is probably necessary to compensate for the small volume of sperm produced. This study also demonstrated that (a) perch sperm motility is of very short duration and sperm velocity rapidly decreases over time, (b) dilution ratio and K^+ and Ca^{2+} concentration as well as osmolality are factors affecting sperm motility parameters, (c) neither calcium nor potassium alone is sufficient to initiate sperm motility; a hypo-osmotic shock is the predominant requirement.

Acknowledgements

We thank Jacky Cosson (CNRS, France) for reviewing the manuscript. This work was supported by GACR no. 524/06/0817, by USB RIFCH no. 6007665809, by NAZV no. QF418 (Czech Republic) and by European Craft project no. COOP-CT-2004, 512629.

References

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes: (I) Effects of pH and temperature. Cell Biol. Int. 29, 101–110.

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A. 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction 128, 819–828.

Alavi, S.M.H., Cosson, J., Kazemi, R., 2006. Semen characteristics in *Acipenser persicus* in relation to sequential stripping. J. Appl. Ichthyol. 22 (Suppl. 1), 400–405.

Alavi, S.M.H., Linhart O., Coward K., Rodina M., 2008a. Fish spermatology: Implication for aquaculture management. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, R. (Eds.). Pp. 397–460. Alpha Science Ltd, Oxford, UK.

Baynes, S.M., Scott, A.P., Dawson, A.P., 1981. Rainbow trout, *Salmo gairdneri*, spermatozoa: Effects of cations and pH on motility. J. Fish Biol. 19, 259–267.

Billard, R., 1983. Effects of ceolomic and seminal fluids and various saline diluents on the fertilizing ability of spermatozoa in the Rainbow trout, *Salmo gairdneri*. J. Reprod. Fertil. 68, 77–84.

Billard, R., Cosson, M.P., 1992. Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122–131.

Billard, R., Takashima, F., 1983. Resorption of spermatozoa in the sperm duct of rainbow trout during the post-spawning period. Bull. Jap. Soci. Sci. Fish. 49, 387–392.

Billard, R., Cosson, J., Crim, L.W., Suquet, M., 1995a. Sperm physiology and quality. In: *Brood stock management and egg and larval quality*, Bromage, N.R., Roberts, R.J. (Eds.). Pp. 25 – 52. Blackwell Science.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995b. Biology of sperm and artificial reproduction in carp. Aquaculture 124, 95–112.

Boitano, S., Omoto, C.K., 1991. Membrane hyperpolarization activates trout sperm without an increase in intracellular pH. J. Cell Sci. 98, 343–349.

Buyukhatipoglu, S., Holtz, W., 1984. Sperm output in rainbow trout (*Salmo gairdneri*). Effect of age, timing, and frequency of stripping and presence of females. Aquaculture 37, 63–71.

Caille, N., Rodina, M., Kocour, M., Gela, D., Flajshans, M., Linhart, O., 2006. Quantity, motility and fertility of tench *Tinca tinca* (L.) sperm in relation to LHRH analogue and carp pituitary treatments. Aqua. Int. 14, 75–87.

Christen, R., Gatti, J.L., Billard, R., 1987. Trout sperm motility: The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. Eur. J. Biochem. 166, 667–671.

Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aqua. Int. 12, 69–85.

Cosson, J., Linhart,O., 1996. Paddlefish (*Polyodon Spathula*) spermatozoa: Effects of potassium and pH on motility. Folia Zool. 45(4), 361-370

Cosson, J., Billard, R., Cibert, C., Dreanno, C., Suquet, M., 1999. Ionic factors regulating the motility of fish sperm. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 161–186. Cache Rive Press, Vienna, IL.

Dietrich, D.J., Kowalski, R., Wojtczak, M., Dobosz, S., Goryczko, K., Ciereszko, A., 2005. Motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa in relation to sequential collection of milt, time of post-mortem storage and anesthesia. Fish Physiol. Biochem. 31, 1–9.

Gatti, J.L., Billard, R., Christen, R., 1990. Ionic regulation of the plasma membrane potential of rainbow trout (*Salmo gairdneri*) sperm: role in the initiation of motility. J. Cell Physiol. 143, 546–544.

Koldras, M., Loir, M., Maisse, G., Le Gac, F., 1996. Study of the composition of seminal fluid and of sperm motility along the genital tract, during a spawning season in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Liv. Resour. 9, 337–345.

Kowalski, R., Wojtczak, M., Glogowski, J., Ciereszko, A., 2003. Gelatinolytic and anti-trypsin activities in seminal plasma of common carp: relationship to blood, skin mucus and spermatozoa. Aquat. Living Resour. 16, 438–444.

Krasznai, Z., Marian, T., Izumi, H., Damjanovich, S., Balkay, L., Tron, L., Morisawa, M., 2000. Membrane hyperpolarization removes inactivation of Ca^{2+} channels leading to Ca^{2+} influx and initiation of sperm motility in the common carp. Proc. Nat. Acad. Sci. 97, 2052–2067.

Krasznai, Z., Morisawa, M., Morisawa, S., Krasznai, Z.T., Tron, L., Marian, T., 2003. Role of ion channels and membrane potential in the initiation of carp sperm motility. Aquat. Living Resour. 16, 445–449.

Krol, J., Glogowski, J., Demska-Zakes, K., Hliwa, P., 2006. Quality of semen and histological analysis of testis in *Perca fluviatilis* L. during spawning period. Czech J. Animal Sci. 51, 220–226.

Kucharczyk, D., Kujawa, R., Mamcarz, A., Skrzypczak, A., Wyszomirska, E., 1996. Induced spawning in perch, *Perca fluviatilis* L. using carp pituitary extract and HCG. Aqua. Res. 27, 847–852.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R., 1995. Fine structure and motility of spermatozoa and composition of the seminal plasma in the perch. J. Fish Biol. 47, 492–508.

Linhart, O., Mims, S.D., Gomelsky, B., Hiott, A.E., Shelton, W.L., Cosson, J., Rodina, M., Gela, D., Bastl, J., 2003. Ionic and osmolality of paddlefish (*Polyodon spathula,* Acipenseriformes) seminal fluid with motility parameters. Aqua. Int. 11, 357–368.

Linhart, O., Rodina, M., Bastl, J., Cosson, J., 2003. Urinary bladder, ionic composition of seminal fluid and urine with charcterization of sperm motility in tench (*Tinca tinca*). J. Appl. Ichthyol. 19, 177–181.

Linhart, O., Rodina, M., Gela, D., Kocour, M., 2004. Optimalization of artificial propagation in European catfish, *Silurus glanis* L. Aquaculture 235, 619–632.

Linhart, O., Slechta, V., Slavik, A., 1991. Fish sperm composition and biochemistry. Bull Inst Zool, Academia Sinica 16, 285–311.

Marshall, W.S., Bryson, S.E., 1988. Evidence for Cl- dependent K^+ secretion by the blood-testis barrier of brook trout. Can. J. Zool. 66, 1603–1609.

Miura, T., Miura, C., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem. 28, 181–186.

Morisawa, S., Morisawa, M., 1986. Acquisition of potential for sperm motility in rainbow trout and chum salmon. J. Exp. Biol. 126, 89–96.

Morisawa, M., Oda, S., Yoshida M., Takai, H., 1999. Transmembrane signal transduction for the regulation of sperm motility in fishes and ascidians. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 149–160. Cache Rive Press. Vienna, IL.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., Yasuda, K., 1983. Effect of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. J. Exp. Biol. 107, 95–103.

Perchec-Poupard, G., Paxion, C., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1998. Initiation of carp spermatozoa motility and early ATP reduction after milt contamination by urine. Aquaculture 160, 317–328.

Perchec, G., Jeulin, C., Cosson, J., Andre, F., Billard, R., 1998. Relationship between sperm ATP content and motility of carp spermatozoa. J. cell Sci. 108, 747–753.

Piironen, J., Hyvarinen, H., 1983. Composition of the milt of some teleost fishes. J. Fish Biol. 22, 351–361.

Rodina, M., Cosson, J., Gela, D., Linhart, O., 2004. Kurokura solution as immobilizing medium for spermatozoa of tench (*Tinca tinca* L.) Aqua. Int. 12, 119–131.

Wirtz, S., Steinmann, P., 2006. Sperm characteristics in perch *Perca fluviatilis* L. J. Fish Biol. 68, 1896–1602.

Sperm motility and monthly variations of semen characteristics in *Perca fluviatilis* (Teleostei: Percidae)

S.M. Hadi Alavi, Marek Rodina, Azadeh Hatef, Vlastimil Stejskal,
Tomáš Policar, Jitka Hamáčková, Otomar Linhart

University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany 389 25, Czech Republic

Abstract

Dynamics of sperm motility (sperm velocity, percentage of motility and beat frequency of flagellum) and monthly variations of semen characteristics (semen volume and osmolality and spermatozoa concentration and motility) were studied in *Perca fluviatilis*. This study showed that spermatozoa velocity, percentage of motility and flagellar beating frequency significantly decrease after activation of sperm motility. Twelve spermiating males were randomly selected and electronically tagged to study monthly variations of semen characteristics. Semen was collected 4 times (29th November 2005, 10th January 2006, 21st February 2006 and 7th April 2006). Semen volume did not change significantly from November to February, but significantly increased in April. Spermatozoa concentration was higher in November and January than February and April. The highest and lowest osmolality of semen was observed in January and April and decreased in February and April. At 15 sec post sperm activation, the lowest percentage of motile spermatozoa was observed in November. The semen samples collected in April showed the lowest motility of spermatozoa (94.64 and 24.3 % at 15 and 30 sec post sperm activation). But, the percentage of motile spermatozoa collected from November to February showed over 65 % motility at 30 sec post sperm activation. At the end of motility period (60 sec post activation), there was not observed motile spermatozoa in April, and the highest was in November. Spermatozoa velocity did not show significant differences at 15 sec post sperm activation. The lowest spermatozoa velocity was observed in April at 30 sec post sperm activation.

Key words: Beat frequency, semen volume, spermatozoa concentration, osmolality, sperm motility

1. Introduction

Percid fishes are candidate for commercial aquaculture because of their economic values as a food fish and many biological characteristics such as accepting formulated feeds and their tolerance to crowding in intensive culture (Kestemont and Melard, 2000). The reproductive cycle of percid fishes is characteristic of annual spawner with group-synchronous development of gonad (Crige, 2000). *Perca* spawn once a year in spring. This annual rhythmus is controlled by exogenous factors such as temperature (Dabrowski et al., 1996; Ciereszko et al., 1998; Migaud et al., 2002) and photoperiod (Migaud et al., 2004)) which are difficult to quantify. To mature, *Perch* need a minimum 160 days

chilling period at a temperature less than 8 ^{o}C. The males become mature at 2 years (Crige, 2000).

The reproductive cycle and sex steroid profiles have been already described in males Eurasian perch (*Perca fluviatilis* L.) (Sulistyo et al., 2000). Gonadal recrudescence in males coincides with the decline in water temperature and the gonadosomatic index increases very rapidly. Androgens (testosterone (T) and 11-ketotestesterone (11-KT)) play key role in early spermatogenesis and spermiogenesis, however it is not declared if 17α, 20β- Dihydroxy-4-pregnen-3-one involve in spermiation (Nagahama, 1994; Miura and Miura, 2003; Vizziano et al., 2008).

It have been already shown that some biological aspects of spermatozoa (such as morphology, ionic composition and osmolality of seminal plasma, spermatocrit, energetic contents (ATP), sperm density and volume as well sperm motility) change during reproductive season, either in freshwater (*Salmo salar m. sebago*, Piironen, 1985; *Cyprinus carpio*, Christ et al., 1996; *Oncorhynchus mykiss*, Koldras et al., 1996; *Barbus barbus*, Alavi et al., 2008b) or marine (*Melanogrammus aeglefinus,* Rideout et al., 2004; *Hippoglossus hippoglossus*, Babiak et al., 2006; *Gadus morhua*, Rouxel et al., 2008) fishes. In freshwater species such as *P. fluviatilis*, spermatozoa are immotile in the seminal fluid due to osmolality and a hypo-osmotic shock is necessary for triggering of initiation of sperm motility (Cosson et al., 1999; Morisawa et al., 1999; Morisawa, 2008). Spermatozoa velocity, percentage of motility and beat frequency of flagellar are main parameters considered in sperm quality assessment (Cosson, 2008; Alavi et al., 2008a).

In *P. fluviatilis*, previous results (Lahnsteiner et al., 1995; Krol et al., 2006; Alavi et al., 2007) showed that (a) the volume of stripped sperm is small in perch ranged from 0.55 ml to 6.78 ml. (b) Spermatozoa concentration is in range of 3.3 – 43.9 $\times 10^{9}$ spermatozoa ml^{-1} (mean, 29 $\times 10^{9}$ spermatozoa ml^{-1}). (c) The motility duration of spermatozoa is very short (less than 1 min). (d) Osmolality of the activation medium affects on sperm motility parameters (sperm motility totally suppressed in activation medium with osmolality higher than 300 mOsmol kg^{-1}). (e) The means of seminal plasma osmolality (mOsmol Kg^{-1}), sodium, chloride, potassium and calcium ions concentrations (mM), are measured as 298, 131, 107, 11 and 2.4 respectively.

In present study, the dynamics of sperm motility parameters (spermatozoa velocity, percentage of motility as well beat frequency of flagella) were studied. Also, the monthly variations of sperm parameters (semen volume and osmolality and spermatozoa density and motility –percentage of motile spermatozoa and sperm velocity) were investigated from November (beginning of spermiating period) to April (the end of spermiation period) in our hatchery condition.

2. Materials and Methods

2.1. Broodfish and sperm collection

To study the dynamics of sperm motility parameters, sperm of 4 spermiating males (total length 198–214 cm, body mass 89–117 g) with initial motility higher than >80 % were used. In case of monthly variations of semen characteristics, twelve spermiating males of *P. fluviatilis* with total length and mass ranging between 14–19 cm and 49–83 g, respectively, were randomly selected from pond at the beginning of

November and then transferred and kept in tank at indoor condition at hatchery. These individuals were electronically tagged for use in this study. Tank of broodfish received a water flow of 10 L min^{-1}, and environmental conditions were controlled at: water temperature 21.5 ± 0.5 °C, oxygen 6.8 ± 0.3 mg O_2 L^{-1} and pH 6.8 ± 0.3. Semen was collected 4 times (29th November 2005, 10th January 2006, 21st February 2006 and 7th April 2006). The males were stripped by pressure on abdomen and by means of a syringe with a needle. No hormone was used for induction of spermiation. We took care to collect all the available semen and to avoid any contamination by urine. Syringes containing semen were kept on ice during analyses.

2.2. Determinations of semen volume and osmolality and spermatozoa density

Semen volume and spermatozoa density were measured by weighting and counting, respectively and according to methods described by Alavi et al., (2007). The semen volume and spermatozoa density were expressed as ml and billion of spermatozoa per ml of semen, respectively. Semen was diluted 1000 times in saline physiological solution. A Burker cells haemocytometer counting chamber was used to count the sperm cells under light microscope (200×). A droplet (10 µl) of the diluted semen was placed on a haemocytometer (depth 0.1 mm) with coverslip. After 10 min (a period to allow sperm sedimentation), the number of spermatozoa was counted in 16 square cells and calculated. The osmolality of semen was measured with a Vapour Pressure Osmometer (Wescor, USA). In this study, the osmolality of semen samples (duplicate per sample) was measured because of low volume of semen to get enough seminal plasma.

2.3. Spermatozoa motility assessments

Spermatozoa motility was evaluated for velocity of spermatozoa (µm s^{-1}) and percentage of motility after activation (%) under 200x magnifications. A 3 CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) was used to record spermatozoa motility. The successive positions of the recorded spermatozoa heads were measured from video frames using a video-recorder (SONY SVHS, SVO-9500 MDP, Japan) and analyzed from five successive frames by micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Spermatozoa velocity of 20 cells approximately measured. Data of spermatozoa velocity are related to motile cells (see Rodina *et al.*, 2008).

2.3.1. Dynamics of sperm motility parameters

A two steps method was used to evaluate spermatozoa motility in this study (Billard and Cosson, 1992). Firstly, semen was diluted in an immobilizing solution (IS) containing NaCl 200 mM, $NaHCO_3$ 2.38 mM (osmolality 380 mOsmol Kg^{-1}) at ratio 1:50. Right after predilution sperm motility were triggered in activation medium containing NaCl or KCl 50–100 mM, Tris 20 mM, pH 8.5 ± 0.5. Measurements of beat frequency were measured using dark field microscopy and stroboscopic illumination. The flagellar beat frequency was measured by reference to the calibrated frequency of the flash illuminator, either on individual sperm cells or on the spermatozoa population

(Cosson, 2008). Measurements on individual spermatozoa indicated that this parameter was not constant for one spermatozoon during the motile phase but decreased progressively (Billard and Cosson, 1992). Therefore, when the beat frequency value of a motile flagellum was determined, it had to be associated with the precise timing of the measurement. The frequency of the flash illuminator was adjusted before each experiment to the highest value previously observed of flagellar frequency (around 60Hz). Then the motility was initiated by dilution and the flash frequency was adjusted until a homogeneous picture of the moving sperm population with immobilized bent flagella was visible.

2.3.2. Monthly variation of sperm motility parameters

The prediluted sperm in IS was activated by dilution in 30 mM NaCl at a ratio 1: 50 (1 µl prediluted sperm: 49 µl activation solution). The adjustable frequency stroboscopic flash illumination was set in 50 Hz for spermatozoa velocity measurement. Spermatozoa motility of each male recorded in *triplicate*. BSA at 0.1% was added to activation solution to avoid stickiness of spermatozoa into the glass slide during motility evaluation.

2.4. Data analysis

All mean values represent mean ± S.E from triplicate. After controlling normality of data by Kolmogrov- Smirnov's test, ANOVA was used for statistical comparisons with Duncan test (SPSS 10.0).

3. Results

3.1. Dynamics of sperm motility parameters

Right after triggering sperm activation, wave(s) present along the flagellum, but its parameters change during motility period (Fig. 1). This study showed that spermatozoa velocity, percentage of motility and flagellar beating frequency significantly decrease after activation of sperm motility ($P < 0.001$, Fig. 2).

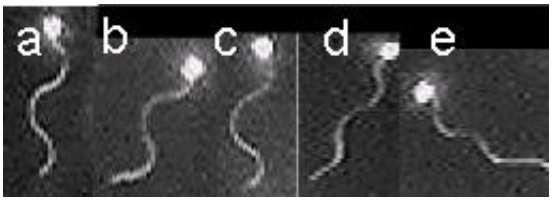

Fig. 1: Motility of spermatozoa of *Perca fluviatilis* after activation in medium with 100mOsmol kg^{-1} at 10(a), 15(b). 35(c), 45(d) and 60(e) s post activation.

3.2. Monthly variations of semen characteristics

Semen volume did not change significantly from November to February, but significantly increased in April (Table 1). Spermatozoa density was higher in November

and January than February and April (Table 1). In contrast, total number of spermatozoa (spermatozoa concentration × semen volume) showed significant increase from beginning (November) to end (April) of spermiation. The highest and lowest osmolality of semen was observed in January and April ($P < 0.05$) and decreased in February and April (Table 1).

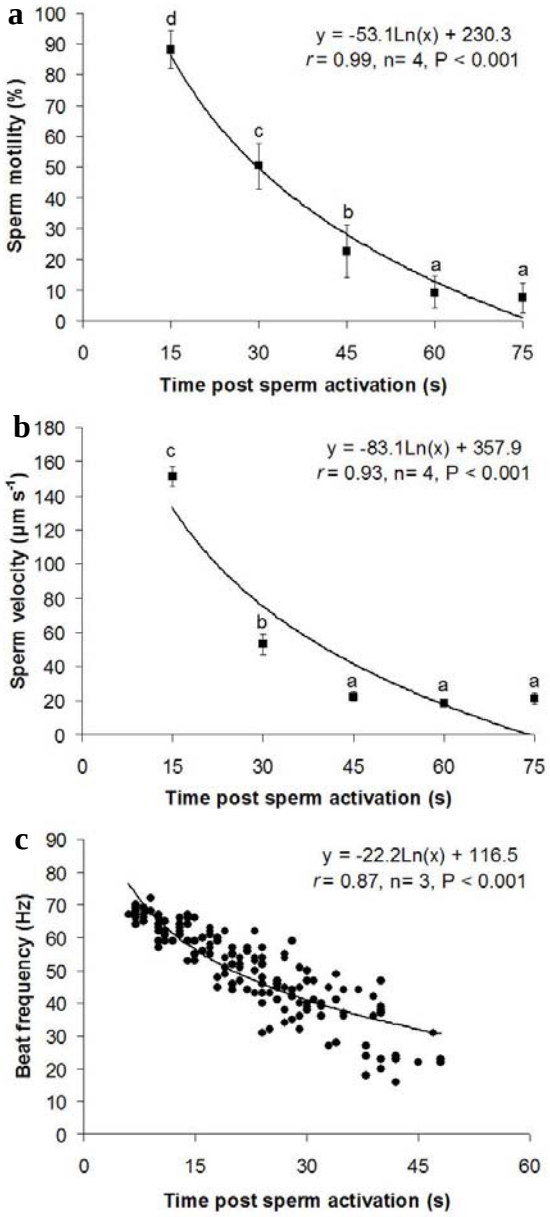

Fig. 2: Percentage of sperm motility (a), spermatozoa velocity (b) and beating frequency of flagella (c) in *Perca fluviatilis* after activation.

Both the percentage of motile spermatozoa and spermatozoa velocity decreased rapidly as a function of time post activation in all sampling times (Fig. 3). Sampling time affected the percentage of motile spermatozoa, significantly ($P < 0.01$). At 15 sec post sperm activation, the lowest percentage of motile spermatozoa was observed in

November (Fig. 3a, $P < 0.05$). The sperm samples collected in April showed the highest rapid decrease in terms of the percentage of motile spermatozoa (the motility of spermatozoa were 94.6 and 24.3 % at 15 and 30 sec post sperm activation). But, the percentage of motile spermatozoa collected from November to February showed over 65 % motility at 30 sec post sperm activation. At the end of motility period (60 sec post activation), there was not observed motile spermatozoa in April, and the highest was in November. Sperm velocity did not show significant differences at 15 sec post sperm activation (Fig. 3b). Again, the lowest spermatozoa velocity was observed in April at 30 sec post sperm activation. Spermatozoa velocity highly decreased from 15 to 30 sec post sperm activation (from >150 μm s^{-1} to >14 μm s^{-1}, respectively), but the differences were low after 30 sec post sperm activation (9.7 μm s^{-1} and 5.8 μm s^{-1} at 45 and 60 sec post sperm activation, respectively).

Table 3. Monthly variations of sperm volume, spermatozoa concentration, total number of spermatozoa and osmolality of the seminal plasma in *Perca fluviatilis*. In each row, values with the same superscript are not significantly different ($P > 0.05$, Duncan test).

Parameter	29th Nov 05	10th Jan 06	21st Feb 06	7th Apr 06
Sperm volume	0.12 ± 0.03^{a}	0.32 ± 0.07^{a}	0.07 ± 0.02^{a}	0.85 ± 0.23^{b}
Spermatozoa concentration	59.17 ± 2.21^{b}	66.53 ± 3.10^{b}	36.01 ± 4.60^{a}	45.45 ± 2.94^{a}
Total number of spermatozoa	9.07 ± 0.06^{b}	22.92 ± 0.20^{c}	2.32 ± 0.09^{a}	38.64 ± 0.64^{d}
Osmolality	423.63 ± 19.93^{b}	523.56 ± 42.09^{c}	373.0 ± 57.68^{ab}	$292.0\pm9.05_{a}$

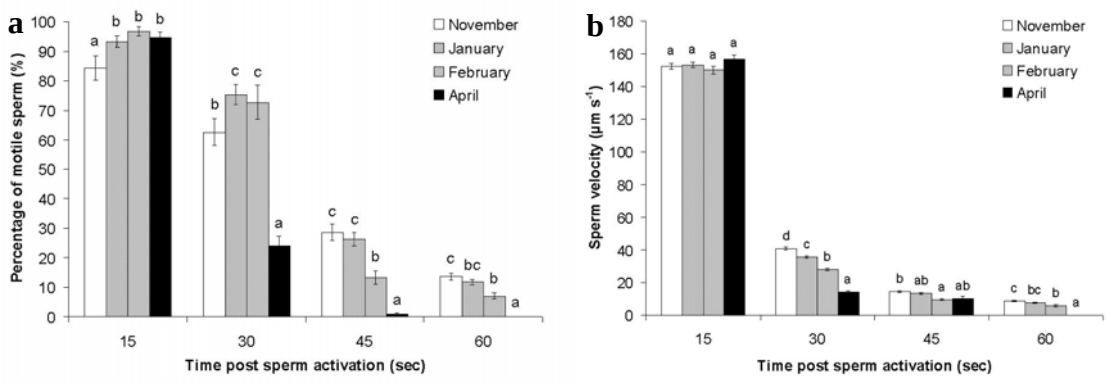

Fig. 3: Monthly changes of percentage of motile spermatozoa (%, a) and spermatozoa velocity (μm s^{-1}, b) in *Perca fluviatilis*. Motility of prediluted sperm in NaCl 200 mM, $NaHCO_3$ 2.38 mM) (osmolality 380 mOsmol Kg^{-1}) was activated in 30 mM NaCl containing BSA 0.1%. Values represent the mean ± S.E from 12 males with three replication per male. Values with the same superscript are not significantly different at the same time post activation ($P > 0.05$, Duncan test).

4. Discussion

4.1. Dynamics of sperm motility parameters

Decreasing of motility parameters of fish spermatozoa have been already demonstrated in several species (see review by Cosson et al., 1999; Alavi and Cosson, 2006). The beat frequency represents the number of waves generated every second. It is directly in proportion to the activity of dyneins and therefore of the rate of ATP

hydrolysis (Cosson et al., 2008). Further studies revealed that their decrease is due to ATP content which is necessary for sperm motility (Perchec et al., 1995, 1998; Cosson et al., 2008). Nevertheless, more studies are needed to find correlations among ATP content, sperm motility parameters and flagellar wave parameters (Alavi et al., 2009). Sperm velocity in perch (185 μm s^{-1}) is lower than pike (*Esox lucius*, 210 μm s^{-1}) (Alavi et al., 2009), Siberian sturgeon (*Acipenser baerii*, 200 μm s^{-1}) (Alavi et al., 2009), but higher than carp (*Cyprinus carpio*, 150 μm s^{-1}) (Linhart et al., 2008) and tench (*Tinca tinca*, 160 μm s^{-1}) (Rodina et al., 2004). This is may be due to differences of sperm morphology (with emphasizes on head shape and flagellar length) and ATP content (Alavi et al., 2009).

4.2. Monthly variations of semen characteristics

In *P. fluviatilis* (Sulistyo et al., 2000), plasma levels of T and 11-KT are very low from April to November. Plasma level of T and 11-KT increase in December and November, respectively. T reaches a peak one month before 11-KT peak in January. Then, the plasma 11-KT concentration highly reduces prior to spawning period in April. Mechanism regulating semen hydration during spermiation plays a major role in determining semen volume in fish (Mylonas et al., 1997; Alavi et al., 2008a,b). Sperm maturation can be induced by increasing the seminal plasma pH in the sperm duct, which leads to increase of intracellular cAMP levels. Sperm maturation is also regulated by DHP. In this study, volume of semen was very low from November to February, but increased significantly in April. It means semen volume is low when plasma T and 11-KT levels are increasing to reach peak, then by decreasing of T and 11-KT level in plasma, the level of DHP increases and spermiation process would be triggered and subsequently increase the volume of semen in April. However, in some teleosts, DHP does not correlate with spermiation, whereas 11KT levels do (Mylonas et al., 1997; Schulz et al., 1994). In addition, in some teleost species an increase of 11-KT have been observed during the first half of the spermiation period (Prat et al., 1990; Jackson et al.,1994; Mylonas et al., 1997). Nevertheless, it has been already declared that semen volume change from the beginning to the end spermiation (reproductive season). In landlocked salmon (Piironen, 1985) an increase has been reported similar to this study, but in *B. barbus* it has been decreased (Alavi et al., 2008b).

Similar to this study, decrease of spermatozoa concentration towards the end of spermiation period has been reported in many teleosts such as, turbot (Suquet et al., 1998), seabass (Dreanno et al., 1999), cod (Rouxel et al., 2008), common barbell (Alavi et al., 2008b), rainbow trout (Munkittrick and Moccia, 1987). But in some species it showed increase such as Atlantic salmon (Piironen, 1985) or Atlantic halibut (Babiak et al., 2006).

Previous studies showed the close correlation between predominant ions of seminal plasma (Na^+, K^+ and Cl^-) and osmolality (Piironen, 1985; Lahnsteiner et al., 1996, 1997; Rouxel et al., 2008). Osmolality of seminal plasma is also related to the "thinning" phenemonon (hydration of semen), which showed high variation among individuals (Morisawa et al., 1979; Koldras et al., 1996; Alavi et al., 2008a,b). Decreasing of osmolality of semen from beginning of spermation (November) to the end (April) is related to hydration of semen that showed increase in this study (semen volume

was significantly higher in April). This is in contrast to the changes of osmolality of halibut sperm that showed significant increase through the reproductive season. In *B. barbus* (Alavi et al., 2008b), the osmolality did not change through the reproductive season. In *G. morhua*, the osmolality of seminal plasma was the highest at the middle of reproductive season, and was lowest either at the beginning or end of reproductive season (Rouxel et al., 2008).

In this study, the percentage of sperm motility was monthly changed that can be related to the osmolality of semen. The percentage of sperm motility highly decreased when the osmolality of semen was significantly lower in April (the end of spermation period). It has been already claimed that the percentage of sperm motility decrease as the reproductive season progressed (for examples in rainbow trout, Munkittrick and Moccia, 1987; in turbot, Suquet et al., 1998; in haddock, Rideout et al., 2004 and in Atlantic halibut, Babiak et al., 2006). However, the percentage of sperm motility did not change in cod (Suquet et al., 2005) and common barbel (Alavi et al., 2008b). Moreover, the percentage of sperm motility can change after short-term storage through spermiating period. Rouxel et al., (2008) found that the percentage of sperm motility of cod peaked during the middle part of the reproductive season after a short-term storage. Our previous study showed that osmolality affects percentage of sperm motility of *P. fluviatilis* (the optimum sperm motility as been observed in 100 mOsmol kg^{-1}). This finding can help us to establish better activation medium for perch spermatozoa at the end of spermiating period (April) by adding Ca^{2+} to activation medium or by increasing the osmolality. Spermatozoa velocity did not change among sampling times at 15 sec post sperm activation, but rapidly decreased at 30 sec post sperm activation (the highest decrease was observed in April). On the other hand, the velocity of spermatozoa in *P. fluviatilis* similar to those of Atlantic halibut (Babiak et al., 2006), cod (Rouxel et al., 2008) and common barbel (Alavi et al., 2008) decrease when the spermiating period is progressed. This is due to ATP decrease which is necessary for sperm movement. At 30 sec post sperm activation, the highest and lowest sperm velocity was observed in November and April. It has been already demonstrated that longer spermatozoa swim faster, in *B. barbus* (Alavi et al., 2008b) and *Salmo salar* (Vladic et al., 2004), due to more ATP available for axonemal beating (Vladic et al., 2004). Taken together, decrease of spermatozoa motility towards the end of spermiating period (either percentage of motility or velocity) probably results from aging process which including changes of sperm morphological parameters (flagellar length) and ATP contents.

In conclusion, sperm of perch should be used immediately after activation to fertilize the eggs due to decrease of motility parameters. In addition, this study showed that semen parameters change in *P. fluviatilis* from beginning (November) to the end (April) of spermiating period. Increase of semen volume can be due to increase of hydration of semen that decreases osmolality of semen and spermatozoa density, but increase total number of spermatozoa. The spermatozoa motility and velocity showed also monthly changes that can be related to the osmolality of semen.

Acknowledgements

This work was supported by projects no. MSM 6007665809, NAZV QF418 and IAA608030801 from Czech Republic.

References:

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1 – 14.

Alavi, S.M.H., Linhart O., Coward K., Rodina M., 2008a. Fish spermatology: Implication for aquaculture management. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, R., (Eds.) Pp. 397–460. Alpha Science Ltd, Oxford, UK.

Alavi, S.M.H., Psenicka, M., Rodina, M., Policar, T., Linhart, O., 2008b. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Teleostei: Cyprinidae) during the reproductive season. Aquat. Living Resour. 21, 75–80.

Alavi, S.M.H., Rodina, M., Policar, T., Kozak, P., Psenicka, M., Linhart, O., 2007. Semen of *Perca fluviatilis* L: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology 68, 276–283.

Alavi, S.M.H., Rodina, M., Viveiros, A.T.M., Cosson, J., Gela, D., Boryshpolets, S., Linhart, O., 2009. Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius* L.). Theriogenology *In press.*

Babiak, I., Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 65, 1587–1604.

Billard, R., Cosson, M.P., 2002. Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122–131.

Christ, S., Toth, G.P., McCarthy, H.W., Torsella J.A., Smith, M.K., 1996. Monthly variation in sperm motility in common carp (*Cyprinus carpio*), assessed using computer-assisted sperm analysis (CASA). J. Fish Biol. 48, 1210–1222.

Ciereszko, R.E., Dabrowski, A., Ciereszko, A., Ottobre, J.S., 1998. Plasma concentrations of steroid hormones in male yellow perch, *Perca flavescens*: the effect of age and photothermal manipulation. Env. Biol. Fish. 51, 97–105.

Craig, J.F., 2000. Percid Fishes: Systematics, Ecology and Exploitation. Blackwell Science, Oxford.

Cosson, J., 2008. Methods to analyse the movements of fish spermatozoa and their flagella. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 63–102. Alpha Science Ltd, Oxford, UK.

Cosson, J., Billard, R., Cibert, C., Dreanno, C., Suquet, M., 1999. Ionic factors regulating the motility of fish sperm. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 161–186. Cache Rive Press. Vienna, IL.

Cosson, J., Groison, A-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008a. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460–486.

Cosson, J., Linhart, O., Mims, S., Shelton, W., Rodina, M., 2000. Analysis of motility parameters from paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) spermatozoa. J Fish Biol. 56, 1348–1367.

Dabrowski, K., Ciereszko, R.E., Ciereszko, A., Toth, G.P., Christ, S.A., El-Saidy, D., Ottobre, J.S., 1996. Reproductive physiology of yello perch (*Perca flavescens*): environmental and endocrinological cues. J. Appl. Ichthyol. 12, 139–148.

Dreanno, C., Cosson, J., Suquet, M., Dorange, G., Fauvel, C., Cibert, C., Billard, R., 1999. Effects of osmolality, morphology and intracellular nucleotid content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. J. Reprod. Fertil. 116, 113–125.

Jackson, L.F., Sullivan, C.V. 1994. Reproduction of White perch: The annual gametogenetic cycle. Trans. Am. Fish. Soc. 124, 563–577.

Kestemont, P., Melard, C., 2000. Aquaculture. In: *Percid Fishes: Systematics, Ecology and Exploitation*, Craig, J.F., (Ed.). Pp. 191–224. Blackwell Science, Oxford.

Koldras, M., Loir, M., Maisse, G., Le Gac, F., 1996. Study of the composition of seminal fluid and of sperm motility along the genital tract, during a spawning season in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Liv. Resour. 9, 337–345.

Krol, J., Glogowski, J., Demska-Zakes, K., Hliwa, P., 2006. Quality of semen and histological analysis of testis in *Perca fluviatilis* L. during spawning period. Czech J. Anim. Sci. 51, 220–226.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R., 1995. Fine structure and motility of spermatozoa and composition of the seminal plasma in the perch. J. Fish Biol. 47, 492–508.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1996. Motility of spermatozoa of *Alburnus alburnus* (*Cyprinidae*) and its relationship to seminal plasma composition and sperm metabolism. Fish Physiol. Biochem. 15, 167–179.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1997. Sperm motility and seminal composition in the Turbot (*Lota lota*). J. Appl. Ichthyol. 13, 113–119.

Linhart, O., Alavi, S.M.H., Rodina, M., Gela, D., Cosson, J., 2008. Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (*Cyprinus carpio*). J. Appl. Ichthyol. 24, 386–396.

Migaud, H., Fontaine, P., Sulistyo, I., Kestemont, P., Gardeur, J.-N., 2002. Induction of out-of-season spawning in Eurasian perch (*Perca fluviatilis*): effects of rates of cooling and cooling durations on female gametogensis and spawning. Aquaculture 205, 253–267.

Migaud, H., Fontaine, P., Kestemont, P., Wang, N., Brun-Bullut, J., 2004. Influence of photoperiod on the onset of gonadogenesis in Eurasian perch (*Perca fluviatilis*). Aquaculture 241, 561–574.

Miura, T., Miura, C., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem. 28, 181–186.

Morisawa, M., 2008. Adaptation and strategy for fertilization in the sperm of teleost fish. J. Appl. Ichthyol. 24, 362–370.

Morisawa, M., Hirano, T., Suzuki, K., 1979. Changes in blood and seminal plasma composition of the mature salmon, *O. keta*, during adaptation. Comp. Biochem. Physiol. 64: 325–329.

Morisawa, M., Oda, S., Yoshida M., Takai, H., 1999. Transmembrane signal transduction for the regulation of sperm motility in fishes and ascidians. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 149–160. Cache Rive Press. Vienna, IL.

Munikittrick, K.R., Moccia, R.D., 1987. Seasonal changes in the quality of rainbow trout (*Salmo gairdneri*) semen: effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma constitutions. Aquaculture 64, 147–156.

Mylonas, C.C., Gissis, A, Magnus, Y., Zohar, Y., 1997. Hormonal changes in male white bass (*Morone chrysops*) and evaluation of milt quality after treatment with a sustained-release GnRHa-delivery system. Aquaculture 153, 301–313.

Nagaham, Y., 1994. Endocrine regulation of gamtogenesis in fish. Int. J. Dev. Biol. 38, 217–229.

Perchec, G., Jeulin, C., Cosson, J., Andre, F., Billard, R., 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747–753.

Piironen, J., 1985. Variation in the properties of milt from the Finnish landlocked salmon (*Salmo salar m. Sebago* Girard) during a spawning season. Aquaculture 48, 337–350.

Prat, F., Zanuy, S., Carillo M., de Mones, A., Fostier, A., 1990. Seasonal changes in plasma levels of gonadal steroids of sea bass, *Dicentrachus labrax* L. Gen. Comp. Endocrinol., 78, 361–373.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. J. Fish Biol. 65, 319–332.

Rodina, M., Cosson, J., Gela, D., Linhart, O., 2004. Kurokura solution as immobilizing medium for spermatozoa of tench (*Tinca tinca* L.) Aqua. Int. 12, 119–131.

Rodina, M., Policar, T., Linhart, O., Rougeot, C., 2008. Sperm motility and fertilizing ability of frozen spermatozoa of males (XY) and neomales (XX) of perch (*Perca fluviatilis*). J. Appl. Ichthyol. 24, 438–442.

Rouxel, C., Suquet, M., Cosson, J, Severe, A., Quemener L., Fauvel, C., 2008. Changes in Atlantic cod (*Gadus morhua* L.) sperm quality during the spawning season. Aqua. Res. 39, 434–440.

Schulz, R.W., Van der Corput, L., Janssen-Dommetholt, J., Goos, H.J.Th., (1994): Sexual steroids during puberty in male Atlantic catfish (*Clarias gariepinus*) serum levels and gonadotropin-stimulated testicular sectretion in vitro. Comp. Biochem. Physiol. 164B, 195–205.

Suquet, M., Dreanno, C., Dorange, G., Normant, Y., Quemener, L., Gaignon, J.L., Billard, R., 1998. The ageing phenomenon of turbot, *Scophthalmus maximus*, spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization and storage capacities. J. Fish Biol. 32, 31–41.

Suquet, M., Rouxel, C., Cosson, J., Severe, A., Quemener, L., Fauvel, C., 2005, Changes in atlantic cod (*Gadus morhua*) sperm quality with time. In: *Larvi 2005*, Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloss, P., (Eds.). Pp. 503–505. European Aquaculture Society, Spec. Publication.

Sulistyo, I., Fontaine P., Rinchard, J., Gardeur, J.-N., Migaud, H., Capdeville, B., Kestemont, P., 2000. Reproductive cycle and plasma levels of steroids in male Eurasian perch, Perca fluviatilis. Aquat. Living Resour. 13, 99–106.

Vermeirssen, E.L.M., Mazorra de Quero, C., Shields, R., Norberg, B., Kime, D.E., Scott, A.P., 2004. Fertility and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*) in relation to dose and timing of gonadotropin-releasing hormone agonist implant. Aquaculture 230, 547–567.

Vizziano, D., Fostier, A., Loir, M., LeFac, F., 2008. Testis development, its hormonal regulation and spermiation induction in teleost fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G., (Eds.) Pp. 103–139. Alpha Science Ltd, Oxford, UK.

Vladic, T.V., Afzelius, B.A., Bronnikov, G.E., 2002. Sperm quality as reflected through morphology in salmon alternative life histories. Biol. Reprod. 66, 98–105.

Chapter 4

Biology of sperm in *Esox lucius* (Teleostei: Esocidae)

Article 6: **Alavi, S.M.H.,** Rodina, M., Viveiros, A.T.M., Cosson, J., Gela, D., Boryshpolets, S., Linhart, O., 2009. Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius* L.). Theriogenology, doi: 10.1016/j.theriogenology.2009.01.015.

Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius* L.)

S. M. Hadi Alavi[1], Marek Rodina[1], Ana T.M. Viveiros[2], Jacky Cosson[3], David Gela[1], Sergei Boryshpolets[1], Otomar Linhart[1]

1- Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in Ceske Budejovice, Vodnany 389 25, Czech Republic
2- Animal Sciences Department, Federal University of Lavras, UFLA, Lavras, MG, 37200-000, Brazil
3- UMR 7009 CNRS, Université Pierre et Marie Curie, Marine Station, 06230 Villefranche sur mer, France

Abstract

Northern pike (*Esox lucius* L.) spermatozoa are uniflagellated cells differentiated into a head without acrosome, a midpiece and a flagellar tail region flanked by a fin structure. Total, flagellar, head and midpiece lengths of spermatozoa were measured and show mean values of 34.5, 32.0, 1.32, 1.17 μm, respectively, with anterior and posterior widths of the midpiece measuring 0.8 and 0.6 μm, respectively. The osmolality of seminal plasma ranged from 228 to 350 mOsmol kg^{-1} (average: 283.88 ± 33.05). After triggering of sperm motility in very low osmolality medium (distilled water), blebs appeared along the flagellum. At later periods in the motility phase, the tip of the flagellum became curled into a loop shape which resulted in a shortening of the flagellum and a restriction of wave development to the proximal part (close to head). Spermatozoa velocity and percentage of motile spermatozoa decreased rapidly as a function of time post activation and depended on the osmolality of activation media ($P < 0.05$). In general, the greatest percentage of motile spermatozoa and highest spermatozoa velocity were observed between 125 and 235 mOsmol kg^{-1}. Osmolality above 375 mOsmol kg^{-1} inhibited the motility of spermatozoa. After triggering of sperm motility in activation media, beating waves propagated along the full length of flagella, while waves appeared dampened during later periods in the motility phase, and were absent at the end of the motility phase. By increasing osmolality, the velocity of spermatozoa reached the highest value while wave length, amplitude, number of waves and curvatures also were at their highest values. This study showed that sperm morphology can be used for fish classification. Sperm morphology, in particular, the flagellar part showed several changes during activation in distilled water. Sperm motility of pike is inhibited due to high osmolality in the seminal plasma. Osmolality of activation medium affects the percentage of motile sperm and spermatozoa velocity due to changes in flagellar wave parameters.

Key words: Esox lucius, Sperm, SEM, Flagella, Osmolality

1. Introduction

Fish spermatozoon is usually differentiated into a head, a midpiece and a flagellum with the typical cylindrical arrangement of “9+2” microtubules. The head

contains the nucleus and therefore the paternal DNA material. Energy required for sperm motility originates from mitochondria, located in the midpiece. Beating of the flagellum itself led to motility of the spermatozoon (Jamieson, 1991; Mattei, 1991; Lahnsteiner and Patzner, 2008). It has been already demonstrated that the family-, species- and subspecies- specific differences regarding the fine structure of spermatozoa can be related to functionality (motility and fertility) spermatozoon (Jamieson, 1991; Mattei, 1991; Cosson et al., 2008a,b; Lahnsteiner and Patzner, 2008) and the physiological/ biochemical characteristics of spermatozoa (Ciereszko et al., 2000; Ciereszko, 2008; Alavi et al., 2008b).

Fish spermatozoa are immotile in the testis (Morisawa, 1985), and acquire the potential for motility during transfer from the testis to the sperm duct (Morisawa and Morisawa, 1986, 1988). It is already well known that the two main factors of seminal plasma preventing the initiation of sperm motility are: its high potassium (K^+) concentrations (in Salmonidae (Billard, 1983; Morisawa et al., 1983a; Stoss, 1983) and in Acipenseridae (Gallis et al., 1991; Toth et al., 1997; Linhart et al., 1995; Alavi et al., 2004)) and its osmolality, either low relative to external medium, which occurs in most marine fishes or high relative to freshwater in non-marine fishes (Morisawa et al., 1983b; Billard et al., 1995a,b; Cosson et al., 2008a). During natural reproduction, fish spermatozoa become motile after discharge into the aqueous environment due to external factors, such as low K^+ concentrations in salmonids or acipenserids, and hypo- or hyper-osmotic shock in freshwater or marine fishes, respectively (Cosson et al., 1999; Morisawa et al., 1999; Alavi and Cosson, 2005, 2006). Cellular studies on the mechanism of sperm motility in fish showed that spermatozoa use external factors as the triggering factor for initiation of the intracellular cascade of events that leads to the initiation of flagellar beating (Cosson et al., 1999; Morisawa et al., 1999; Morisawa and Yoshida, 2005). Sperm motility is generated by a highly organized microtubule-based scaffolded structured called the axoneme (Inaba, 2003, 2008).

Because motility of sperm in fish is influenced by several external factors such as pH, temperature, ions and osmolality (Cosson et al., 1999; Alavi and Cosson, 2005, 2006; Alavi et al., 2007, 2008b), understanding effects of these factors are helpful in improving methods of artificial reproduction and provides information for developing better short- and long-term storage (cryopreservation) conditions for sperm (Linhart et al., 2004; Alavi et al., 2007; Rodina et al., 2007). In addition, it leads to comparative knowledge regarding species – specific differences in motility of sperm (Morisawa et al., 1983b; Cosson et al., 2008a; Alavi and Cosson, 2006).

Specific studies on sperm motility in terms of flagellar beating and wave parameters have led to a better understanding of the internal mechanics explaining how the movement characteristics of sperm flagella are established (Ishijima et al., 1998). For this purpose, fish spermatozoa are extremely suitable because they have remarkable variations in both structure and function (Ishijima et al., 1998; Cosson et al., 2008a,b; Lahnsteiner and Patzner, 2008). Most knowledge on sperm movement developed by simple flagella comes from studies on sea urchin (echinoids) sperm (Cosson, 2004) which are long term swimmers (tens of hours). Nevertheless, fish spermatozoa show several original features : 1- their duration of motility is very short (Billard and Cosson, 1992), 2- their ability to immediately initiate motility upon contact with the external medium, 3- the specific differences which are observed between freshwater and marine

species (Cosson et al., 1999; Morisawa et al., 1999; Cosson, 2004) and 4- the variety in flagellar motility patterns exhibited among species and/or conditions (Cosson et al., 2000; Alavi et al., 2008a). The flagellar movement of fish spermatozoa may be classified into two groups according to sperm structure (Ishijima et al., 1998); (1) spermatozoa having elongated mitochondria and (2) spermatozoa having a simple structure with rudimentary mitochondria. The first and second type appears in fish using internal and external fertilization strategies, respectively.

The northern pike *(Esox lucius* L.*)* is a freshwater species inhabiting the northern hemisphere which has been cultivated extensively in Europe and Asia since the middle ages (Steinberg, 1992). Males and females become sexually mature at age 2-3 and 3-4 years, respectively, and spring spawning occurs in the shallow waters when the temperature of this water reaches 4-7 °C (Kouril and Hamackova, 1975). A few studies have been published on sperm biology in pike. These studies have shown that the stripped sperm density ranges $7 – 22 \times 10^9$ spermatozoa ml^{-1} and the total number of spermatozoa is $10 – 15 \times 10^9$ (Koldras and Moczarski, 1983; Babiak et al., 1995, 1997). Moreover, the ionic composition and osmolality of seminal fluid are Na^+ (116 mM), Cl^- (116 mM) and K^+ (25 mM), Ca^{2+} (1 mM) and 273 mOsmol kg^{-1} (44,45). Pike spermatozoa have a very short duration of motility, up to a maximum of 90 sec in freshwater at 4^0C (temperature of activation medium) (Linhart, 1984; Babiak et al., 1995, 1997; Hulak et al., 2008a,b).

In the present study, the main objective was to investigate the effects of osmolality on: (a) sperm morphology during activation, (b) percentage of motile sperm and (c) sperm velocity. Various flagellar wave parameters (wave length, wave amplitude, number of waves and tracks curvatures) were also determined as a function of the osmolality of the activation media.

2. Materials and methods

2.1. Sample collection

During the spawning season in March in Vodnany, Czech Republic, 16 mature male pike (total length 50 – 67 cm; body weight 912 – 1400 g) were captured from large natural ponds. After transportation to the hatchery, sperm samples were collected by abdominal massage. Hormonal induction was not used for sperm maturation. All attempts were made to avoid contamination of sperm by urine, mucus or water during stripping. Sperm samples were collected in syringes and kept in an ice box ($0 – 2\ ^0$C) during transportation to the laboratory and during the motility analysis assays. Sperm motility was assessed at room temperature under the microscope ($18 – 20\ ^0$C).

2.2. Osmolality effects on sperm morphology

To study the effects of hypo-osmolality on sperm morphology, the motility of spermatozoa was first activated in distilled water containing 0.1% BSA (15 mOsmol kg^{-1}) under dark-field or phase contrast microscopy with an objective lens of 100x magnification. Samples from the same males were collected at 45 and 60 sec post activation and used for observations using scanning electron microscopy (SEM). For

SEM, sperm motility was activated only in distilled water without BSA (3 mOsmol kg^{-1}). BSA usually uses for avoiding stickiness of sperm into the slides under microscopy observation. The samples of sperm were directly fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and post fixed and washed repeatedly for 2 h at 4°C in 4% osmium tetroxide followed by dehydration through an acetone series. The samples were coated with gold under vacuum for SEM with a Coating Unit E5100 (Polaron Equipment Ltd., England) and observed by use of a JSM 6300 SEM (JEOL Ltd., Akishima, Tokyo, Japan). Morphological parameters were then measured from micrographs using the Olympus MicroImage software (version 4.0.1. for Windows).

2.3. Osmolality of seminal plasma and activation media

The osmolalities of seminal plasma and activation media were measured using a Vapour Pressure Osmometer (USA). Sperm samples were centrifuged first at 3000 rpm for 3 min, followed by a second centrifugation at 10 000 rpm for 10 min and the supernatant was used for measurement of the osmolality of seminal plasma.

2.4. Osmolality effects on sperm motility and flagellar wave parameters

The effects of osmolality on the percentage of motile spermatozoa and spermatozoa velocity were studied in activation media composed of different concentrations of NaCl, sucrose and mannitol in distilled water containing 20 mM Tris-HCl at pH 8.5. Distilled water was used as a control. In all the media used, BSA was added at 0.1% (w/v) to avoid stickiness of sperm to the glass slides. For activation, 0.2 – 0.5 µl of sperm were directly mixed within a 49 µl of activation media. The motility was recorded (including a time reference) immediately after activation using a 3 CCD video camera mounted on a dark-field microscope under stroboscopic light. Sperm motility (velocity and the percentage of motile spermatozoa) and flagellar waveform (number of waves and curvatures, wave length and wave amplitude) parameters were analyzed on successive video frames by a micro image analyzer (Cosson et al., 2000; Alavi et al., 2008d). Mean velocity was based on motile spermatozoa only. The wave length was measured as a distance between repeating units of a propagating wave. Peak-to-peak amplitude was measured as the distance between two successive peaks within one sine wave. Number of waves and number of curvatures per flagellum were counted from prints of enlarged video frames. It should be noted that the sperm motility pattern in pike is symmetric (close to a true sine shape); therefore, the use of peak amplitude and wave length is simple and unambiguous for reliable measurement.

2.5. Data analysis

The results are shown as mean ± standard deviation (S.D.). After controlling normality of data by Kolmogrov- Smirnov's test, statistical significance was tested using analysis of variance (ANOVA, SPSS 10.0) with Duncan test. Probability values < 0.05 were considered significant.

3. Results

3.1. Sperm morphology

The northern pike spermatozoa are uniflagellated and are differentiated into a head without acrosome, a midpiece with a cylindrical shape and a tail region called a flagellum presenting one lateral fin (Fig. 1). Table 1 shows inter-specimen differences of the morphological parameters measured by SEM.

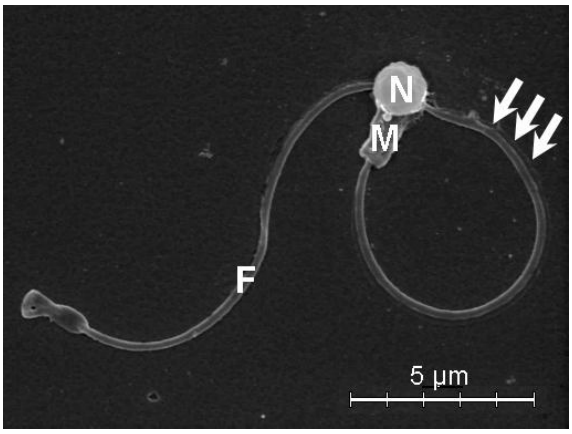

Fig. 1: Sperm morphology in *Esox lucius* using scanning electron microscopy. N: Nucleus, M: Midpiece, F: Flagellum. Arrows show fin along flagellum of spermatozoa.

Table 1: Morphological parameters of *Esox lucius* spermatozoa using scanning electron microscopy (SEM). Data are presented as mean ± standard deviation (n = 3). Number of spermatozoa per each male is shown in parentheses.

Parameter	**Male 1** (58)	**Male 2** (67)	**Male 3** (67)	**Total** (n= 3)
Head length	1.32 ± 0.12^{a}	1.31 ± 0.14^{a}	1.29 ± 0.16^{a}	1.30 ± 0.14
Head width	1.49 ± 0.12^{b}	1.38 ± 0.10^{a}	1.39 ± 0.10^{a}	1.40 ± 0.11
Midpiece length	1.17 ± 0.36^{b}	0.91 ± 0.26^{a}	1.07 ± 0.30^{b}	1.04 ± 0.32
Anterior width of midpiece	0.75 ± 0.15^{a}	0.71 ± 0.14^{a}	0.72 ± 0.17^{a}	0.73 ± 0.16
Posterior width of midpiece	0.56 ± 0.11^{b}	0.51 ± 0.12^{a}	0.56 ± 0.12^{b}	0.54 ± 0.12
Flagellar length	32.01 ± 3.41^{b}	29.60 ± 4.71^{a}	29.03 ± 3.36^{a}	30.12 ± 4.08
Total length	34.49 ± 3.40^{b}	31.80 ± 4.73^{a}	31.38 ± 3.37^{a}	32.47 ± 4.12

In each parameter, values with similar letters are not statistically significant ($P > 0.05$).

3.2. Osmolality effects on sperm morphology

Observations of spermatozoa after activation in distilled water using dark-field (Fig. 2A), phase contrast microscopy (Fig. 2B) and SEM (Fig. 2C) showed that, almost 30 sec after triggering of sperm motility, blebs appeared along the flagellum and these blebs prevented correct and efficient wave propagation. The tip of the flagellum became curled into a loop shape which shortened the flagellum; also the middle part of the flagellum became folded around itself which shortened the efficient length of the flagellum. Further damages appeared which could be specific to pike spermatozoa and related to the presence of a fin structure along thwe flagellum. In contrast, such folding,

blebs or damages were not seen in flagella when activation of spermatozoa was triggered in higher osmolality media (above 125 mOsmol kg^{-1}) (Fig. 3).

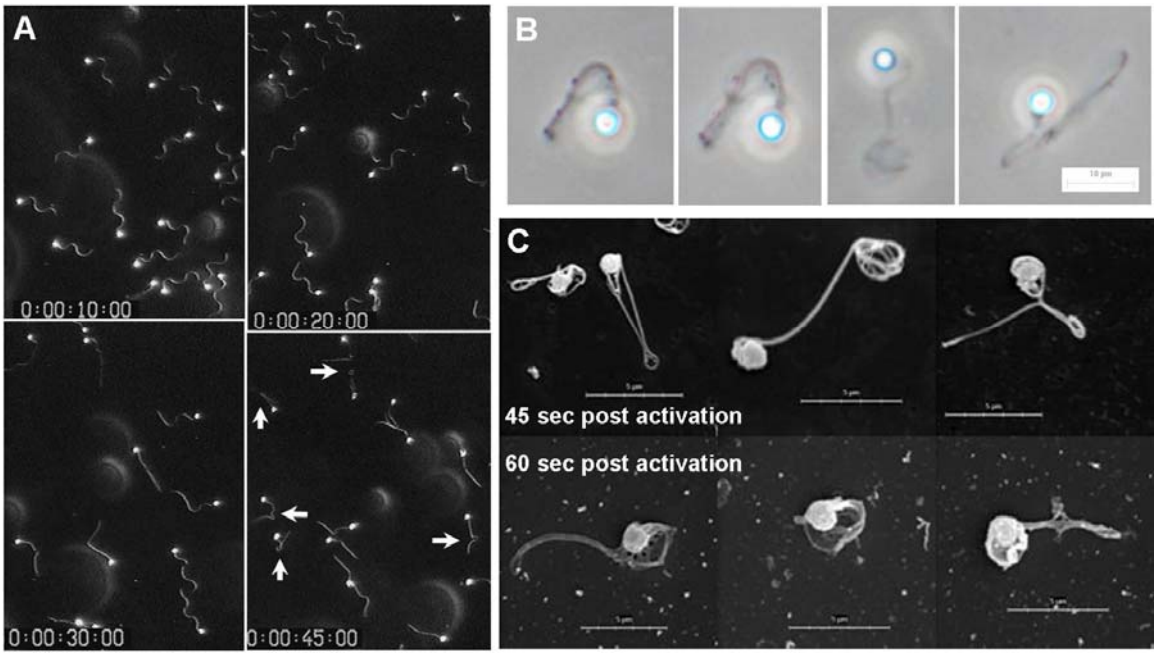

Fig. 2: Effects of hypo-osmotic shock on sperm morphology of *Esox lucius* under dark field microscopy with stroboscopic illumination (A), phase contrast microscopy (B) and scanning electron microscopy (C) at 45 and 60 s post activation of spermatozoa in distilled water. Scale bars for micrographs are 5 µm.

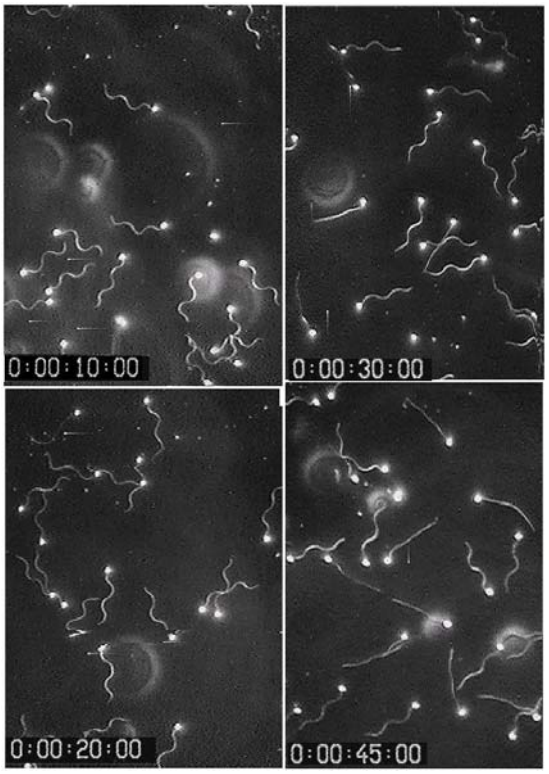

Fig. 3: Motility of sperm of *Esox lucius* in NaCl medium at 235 mOsmol kg^{-1} under dark field microscopy with stroboscopic illumination.

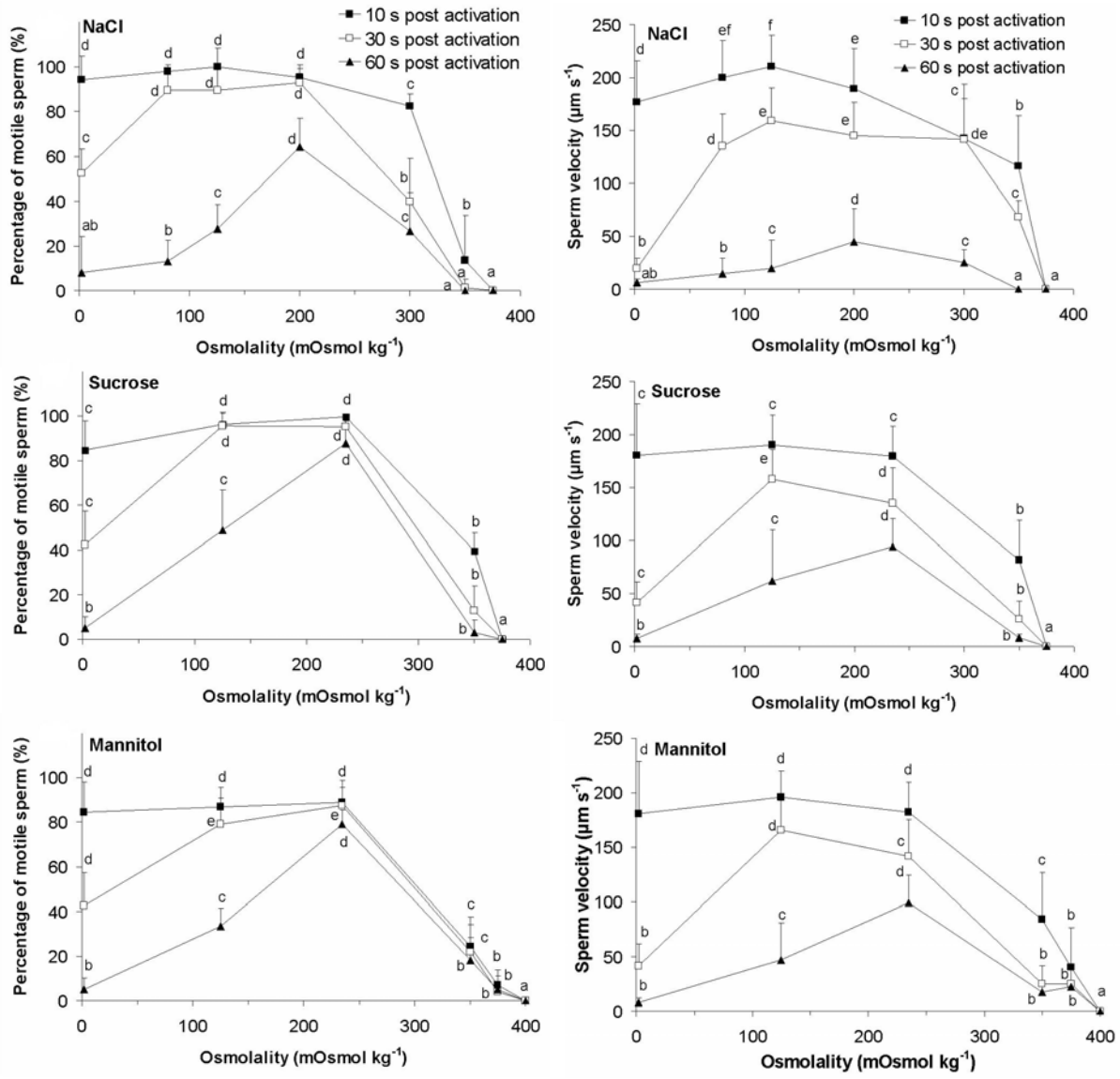

Fig. 4 (Left): Effects of osmolality on percentage of motile spermatozoa of *Esox lucius* after activation in media containing NaCl (n= 5), sucrose (n= 3) and mannitol (n= 3). All solutions were buffered using 20mM Tris and pH adjusted to 8.5. The mean values of osmolality (mOsmol kg^{-1}) of the seminal plasma were 288.50 ± 36.05 (232 – 349) (NaCl) and 272.33 ± 44.26 (227 – 324) (sucrose and mannitol). Data are expressed as mean ± standard deviation. Values with the same letters are not significantly different ($P > 0.05$).

Fig. 5 (Right): Effects of osmolality on spermatozoa velocity of *Esox lucius* after activation in media containing NaCl (n= 5), sucrose (n= 3) and mannitol (n= 3). All solutions were buffered using 20mM Tris and pH adjusted to 8.5. The mean values of osmolality of the seminal plasma were 288.50 ± 36.05 (232 – 349) mOsmol kg^{-1} (NaCl) and 272.33 ± 44.26 (227 – 324) mOsmol kg^{-1} (sucrose and mannitol). Data are presented as mean ± standard deviation. Values with the same letters are not significantly different ($P > 0.05$).

3.3. Osmolality of the seminal plasma

The osmolality of seminal plasma of sampled males in this study was in the range of 228 – 350 (average 283.88 ± 33.05) mOsmol kg^{-1}.

3.4. Osmolality effects on sperm motility (percentage of motile spermatozoa and sperm velocity)

The percentage of motile spermatozoa depended on the osmolality of the activation medium and changed as a function of time post activation ($P < 0.01$). The maximum percentage of motile spermatozoa was observed at 10 s post activation, for osmolality values ranging 125 to 235 mOsmol kg^{-1} either after activation in NaCl, sucrose or mannitol (Fig. 4). Except of sucrose as activation medium, there were no significant differences between osmolalities 0.0 and 200 in NaCl or 0.0 and 235 in mannitol ($P > 0.05$). At 30 s post activation, the percentage of motile sperm was significantly lower in distilled water and in higher osmolality (>300 mOsmol kg^{-1}) than sperm in the osmolality range of 80 – 235 mOsmol kg^{-1}) ($P < 0.05$). At 60 s post activation, the percentage of motile spermatozoa (60 to 85%) was observed to be the highest at 200 – 230 mOsmol kg^{-1} ($P < 0.05$). The percentage of motile sperm was close to 0 % both in distilled water and very high osmolality conditions. Activation of sperm motility was totally suppressed at 375 mOsmol kg^{-1} in NaCl or sucrose and at 400 mOsmol kg^{-1} in mannitol.

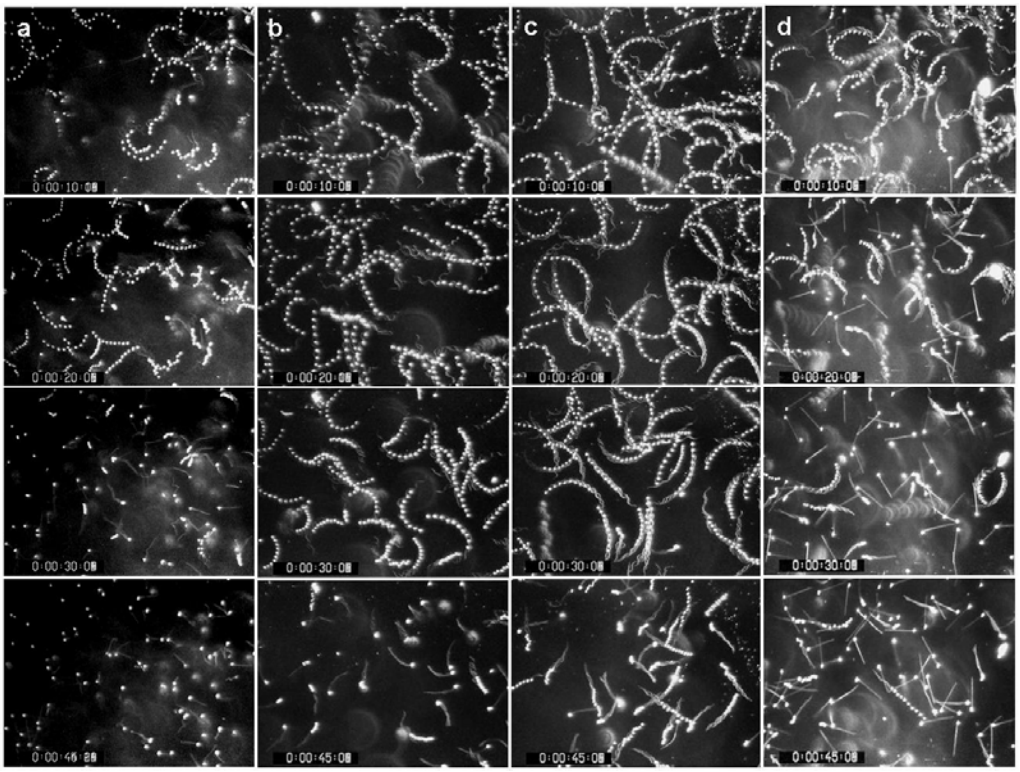

Fig. 6: Sperm head trajectories in *Esox lucius* during motility after activation in (a) distilled water (0.0 mOsmol kg^{-1}) or in (b, c or d) activation media containing NaCl + 20 mM Tris, pH 8.5 at osmolalities values of (b) 50, (c) 200 and (d) 300 mOsmol kg^{-1} respectively.

The highest spermatozoa velocity was observed when osmolality was in the range of 125 – 235 mOsmol kg^{-1} at 10 s post activation after triggering motility in NaCl, sucrose or mannitol (Fig. 5) ($P < 0.05$). Velocity of spermatozoa was significantly higher after activation in NaCl as compared to sucrose and mannitol at the same osmolality value ($P < 0.05$). Spermatozoa velocity decreased significantly as a function of time post activation at the same osmolality ($P < 0.01$). At 30 s post activation, the sperm velocity was highest at 125 – 200 mOsmol kg^{-1} and lowest at high osmolality (> 350 mOsmol kg^{-1}) or in distilled water. At 60 s post activation, spermatozoa velocity was the highest at 235 mOsmol kg^{-1} and was significantly lower in distilled water or in high osmolality medium ($P < 0.01$).

Sperm head trajectories exhibit major differences in relation to the osmolality of activation medium. At the same time post sperm activation, the trajectory distance increased by increasing the osmolality of activation media (Fig. 6). Just after activation, spermatozoa exhibit circular trajectories independent of osmolality of activation medium. However, the diameter of trajectory increases by increasing osmolality of the activation medium. At later period of sperm motility (30 s post activation), trajectory becomes more linear (circles with much larger diameter) in distilled water. Linear trajectory was observed at 45 s post sperm activation in range 50 – 300 mOsmol kg^{-1}.

3.5. Osmolality effects on flagellar wave parameters during sperm motility

After triggering of sperm motility in activation media, beating waves propagated along the full length of flagella, while waves appeared dampened at later period in the motility phase, and even completely absent at the end of the motility phase (Fig. 7). In addition, the wave parameters change during the flagellar activity period and vary according to osmolality of the activation media. Flagellar wave parameters during sperm activation at different osmolalities are shown in Figure 8. By increasing osmolality, the velocity of spermatozoa reaches the highest value, while concomitantly, length and amplitude of waves showed the highest significant values as well as the largest number of waves and curvatures ($P < 0.05$). At the same osmolality of activation media, wave length and amplitude were decreased, but number of curvatures and waves were increased as a function of time post activation.

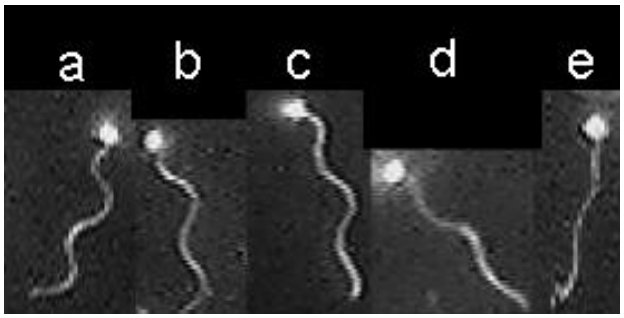

Fig. 7: Sperm motility of *Esox lucius* spermatozoa in activation media containing NaCl with osmolality values of 100 mOsmol kg^{-1} at 8 (a), 20 (b), 30 (c), 45 (d) and 60 (e) s post activation.

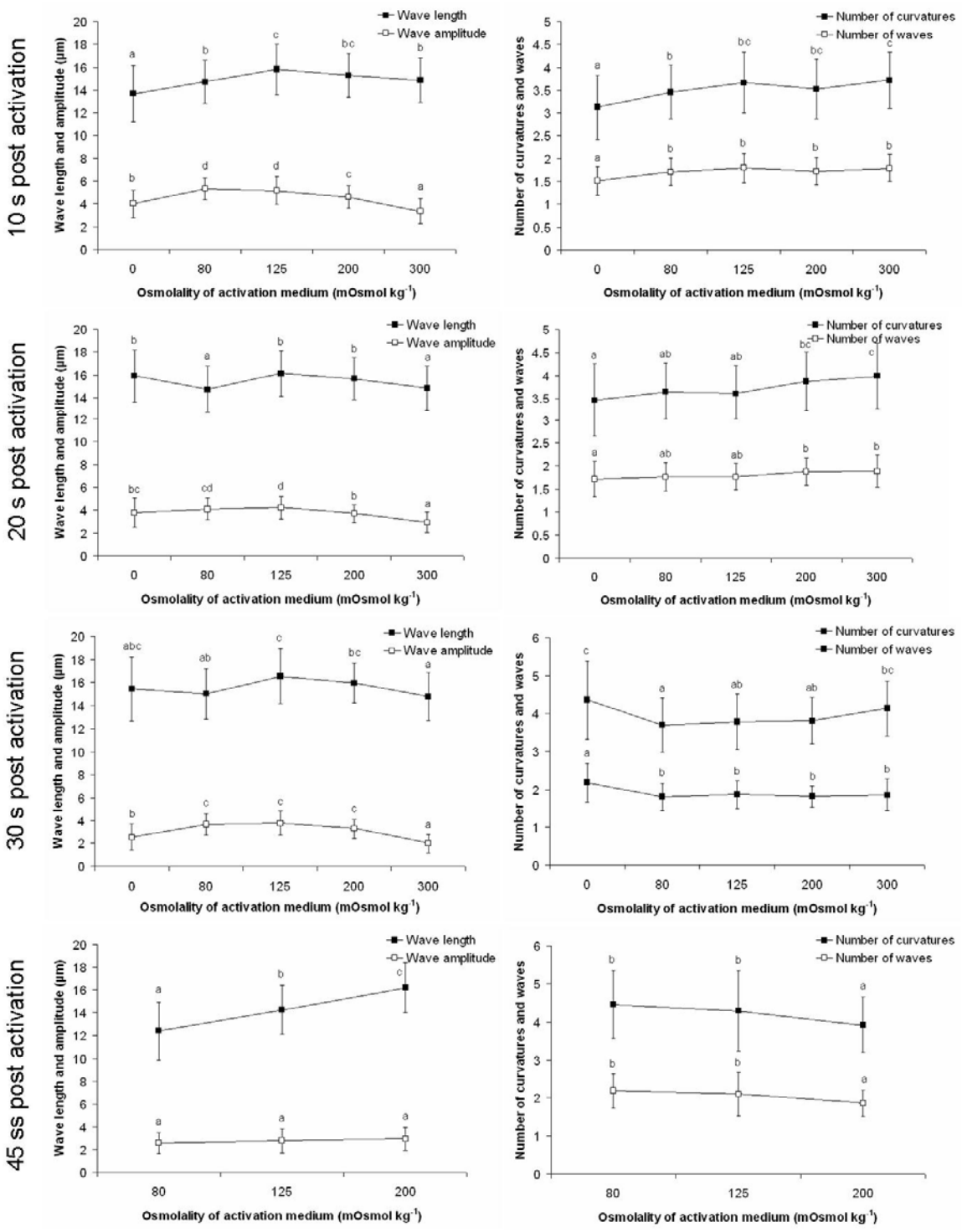

Fig. 8: Flagellar wave parameters during sperm motility in *Esox lucius* at different osmolalities after activation in media containing NaCl + Tris-HCl 20mM, pH 8.5 at 10, 20, 30 and 45 s post activation. The mean value of osmolality of the seminal plasma was 288.50 ± 36.05 (232 – 349) (mOsmol kg^{-1}). Values with the same letters are not significantly different ($P > 0.05$).

Table 2: Comparison of some morphological parameters of spermatozoa between *Esox lucius* and some teleost fishes. Numbers in brackets are references.

Species	Shape of head	Width of head	Length of head	Length of midpiece	Number of mitochondria	Length of flagellum	Total length	Presence of fin along flagellum
Esocidae								
Esox lucius [1]	roundish	1.5-1.8	2.0		1		37.42	Yes
E. niger [2]		1.86	1.84				31.3	
Esox lucius [this study]	roundish	1.40	1.30	1.04		30.12	32.47	Yes
Perciformes								
Perca fluviatilis [3]	ovoid	1.78	1.87	0.87	1	30-35		No
Sarotheron melanotheron [4]	roundish	1.65	1.4-1.5	0.50-0.61		12.5-14.8	14.4-16.5	No
Cyprinidae								No
Cyprinus carpio [5]	roundish	2.5	3.3		7-9	43		No
Tinca tinca [6]	roundish	1.71	1.27	0.86	2-6	25.45	26.1	No
Barbus barbus [7]	roundish	1.80	1.71	0.48	4-6	54.30	56.35	No
Cottidae								
Cottus gobio [8]	elongated	0.79	2.24	1.98	5-6			No

[1] Billard, 1970; [2] Linhart et al., 1991; [3] Lahnsteiner et al., 1995; [4] Alavi et al., unpublished data; [5] Billard et al., 1995b; [6] Psenicka et al., 2006; [7] Alavi et al., 2008c; [8] Lahnsteiner et al., 1997.

4. Discussion

4.1. Sperm morphology

In Esociformes, only the spermatozoa of *E. lucius* (Billard, 1970), chain pickerel (*Esox niger*) (Linhart et al., 1991) and muskellunge (*Esox masquinongy*) (Lin et al., 1996) have been briefly described, but morphological parameters have not been quantitatively determined. In all three species, spermatozoa are uniflagellated, acrosomeless and have clearly differentiated head, midpiece and flagellum. Similar to *E. masquinongy*, the *E. lucius* spermatozoon have a spherical head, 1.40 µm in diameter. The midpiece is elongated in both pike and muskellunge. In *E. lucius* but not *E. masquinongy*, a cytoplasmic expansion (fin) is present on one side of the flagellum, slightly different of those that have been demonstrated in salmonid spermatozoa (Ishijima et al., 1998; Lahnsteiner and Patzner, 2008) or sturgeons (Psenicka et al., 2008) where fins are present on both sides. Billard (1970) commented that *E. lucius* spermatozoon showed a strong resemblance to the aquasperm of Cyprinidae (Billard et al., 1995) and were similar to sperm of Perciformes such as perch, *Perca fluviatilis*, and tilapia, *Oreochromis* spp. Stein (1981), in contrast, found pike sperm is similar to the sperm of *Perca* and *Cottus*, in head from. A summary of these data is given in Table 2. *E. lucius* sperm resembles that of tench, *Tinca tinca* (Cyprinidae) and *P. fluviatilis* (Percidae) in terms of head shape and morphological parameters (Lahnsteiner et al., 1995; Psenicka et al., 2006), but differ in mitochondria number. Pike spermatozoon has the same head form as tilapia, but values of morphological parameters seem to be higher (Alavi et al., unpublished data). Pike spermatozoa highly differ from those of *Cottus gobio* (Lahnsteiner et al., 1997); in cases of width and length of head, head form, length of midpiece and number of mitochondria (Table 2). Compared to *Barbus barbus*, a cyprinid species, *E. lucius*, spermatozoa have shorter flagella and total length, and the other morphological parameters also differ (Alavi et al., 2008c). As a conclusion, spermatozoa of Esocidae species are strongly distinguishable from those of other families and orders, which represents good characteristics for fish phylogeny and systematic.

4.2. Osmolality effects on sperm morphology

In both freshwater and marine fishes, exposure of sperm to distilled water (hypo-osmotic shock) has led to different types of flagellar damages after activation. The motility duration of fish spermatozoa can be limited by flagellar damages appearing during the motility. In both freshwater and marine fishes, two main and common flagellar damages were reported (Cosson et al., 2000, 2008a,b); (a) cytoplasmic blebs along flagellar length during the motility period which impair the propagation of wave and (b) curling structure at flagellar tip particularly close to the end of motility period, which shortens obviously the flagellar length and leads to decrease efficient axonemal beating. Damages such as blebs and curling usually result from local membrane defects caused mainly hypo-osmotic shock. Therefore, these damages are readily encountered in sperm samples contaminated by urine (Perchec-Poupard et al., (1998). Nevertheless, it is known that local swelling of spermatozoa membranes and flagellar microtubules curling or looping at the flagellar tip can be reversed after exposure of fish spermatozoa to high-

osmotic conditions (isotonic to seminal plasma) (Cosson et al., 2008a,b; Linhart et al., 2008). This is paralleled by the recovery of the ATP content of the spermatozoa which allows a second run of sperm motility (called reviving) (Billard et al., 1995; Rodina et al., 2004). In the present study, we have described a behavior specific to pike spermatozoa which has not been observed in any other fish species. Close to the end of the motility period, the mid part of the flagellum turns and rotates around itself where a fin is located on one side of the flagellum.

4.3. Osmolality of the seminal plasma

The osmolality (mOsmol kg^{-1}) of the seminal fluid in the present study (283 ± 33) was slightly higher than that observed in our previous study (273 ± 22) (Hulak et al., 2008a,b); however, both values are similar to the osmolality observed for muskellunge sperm (289.5 ± 16.8) (Lin et al., 1996). The slight difference observed can be related to the ionic composition of the seminal plasma, which depends on the period of the year for sperm collection (spermiation period) (Billard et al., 1995a; Alavi et al., 2008b). The osmolality of pike seminal plasma is slightly lower than observed for marine fish species (310 - 415 mOsmol kg^{-1}), but similar to freshwater species as perch and carp (284 and 286 mOsmol kg^{-1}, respectively) or slightly higher (see for example tench, and Atlantic salmon, *Salmo salar* 230 and 245 mOsmol kg^{-1}, respectively) in any case, it is obviously higher than in sturgeons (maximum 100 mOsmol kg^{-1}). These differences are mainly related to the ionic and biochemical composition of the seminal plasma which besides being species-specific and also vary according to the advancement in the reproductive season and other factors such as aging of spermatozoa, sperm density and others (see reviews Billard et al., 1995a; Alavi and Cosson, 2006; Alavi et al., 2008b).

4.4. Osmolality effects on sperm motility (percentage of motile spermatozoa and sperm velocity)

After activation of sperm in distilled water, the percentage of motile sperm and sperm velocity showed higher values in the present study compared to our previous results (Hulak et al., 2008a,b). Variations in sperm motility within individuals (Babiak et al., 1995), is known to exist and depends on several parameters such as energetic content and metabolism of the spermatozoa or aging of sperm through the reproductive season (Alavi et al., 2008b). Therefore, it is of interest to further studies for understanding sperm motility as indicators in relation to seasonality. Taken together, *E. lucius* spermatozoa behave similarly to those of other fish species (Alavi and Cosson, 2005, 2006; Cosson et al., 2008a,b; Alavi et al., 2008b): they present a short period of motility and motility parameters of spermatozoa rapidly decrease as a function of time post sperm activation.

Our study is the first to illustrate the effects of osmolality on sperm motility of *E. lucius* spermatozoa. Lin and Dabrowski (1996) showed that an osmolality >340 mOsmol kg^{-1} (in activation media containing either electrolyte such as NaCl or KCl or glucose) totally suppressed muskellunge spermatozoa motility. The same was observed for pike in the present study. However, the best osmolality value for motility of pike spermatozoa ranged from 125 – 235 mOsmol kg^{-1}, in which > 60% of spermatozoa were motile. Similar effects of osmolality have been reported in other teleost fishes such as common

carp (Cosson et al., 1991), tilapia (Legendre, Cosson, Alavi, Linhart, unpublished data) and perch 9Lahnsteiner et al., 1995; Alavi et al., 2007). The optimal osmolality (in mOsmol kg^{-1}) for sperm motility is 90 – 110 in common carp, 100 – 300 in tilapia and 100 – 150 in perch. However, Lin and Dabrowski (1996) indicated that, in muskellunge, K^+ ions prolonged the motility period of spermatozoa at the same concentrations of NaCl (100 mM). As a general conclusion, a hypo-osmotic shock triggers the initiation of pike spermatozoa motility in a fashion similar to those observed for muskellunge and other freshwater species (Lin and Dabrowski, 1996; Morisawa et al., 1999; Morisawa and Yoshida, 2005; Alavi and Cosson, 2006). On the other hand, osmolality of the seminal plasma inhibits the motility of E. *lucius* spermatozoa (Hulak et al., 2008a). But, potentiality for sperm motility and optimum osmolality for observation of the highest motility parameters seems to be variable within species. We suspect that K^+ ions play a main role in the activation of pike sperm, because the motility and behavior of spermatozoa are very similar to those of cyprinids. It has been confirmed that the motility of common carp sperm is highly decreased or suppressed in presence of K^+ channel inhibitors (Krasznai et al., 2000). In our present study, sperm velocity of pike was slightly higher than that observed in our previous results 180 vs. 160 μm s^{-1} (Hulak et al., 2008a). This small difference may be related to the ionic composition of the seminal plasma (Na^+ and Cl^- contents) or male body weight. It has been shown that a highly significant correlation exists between sperm velocity and Na^+, Cl^- contents in the seminal plasma and body weight (Hulak et al., 2008b). However, *E. lucius* sperm velocity is higher than those of carp (Linhart et al., 2008), perch (Lahnsteiner et al., 1995; Alavi et al., 2007), tench and tilapia (Alavi et al., 2008a); 210 vs. 150, 185, 160 and 50 – 80 μm s^{-1} respectively. The sperm velocity seems to be similar to that of sturgeon [50]. Sperm velocity of spermatozoa depends on several parameters such as head dimension (diameter of head), beat frequency, length of flagellum, physical parameters of wave propagation such as wave length and amplitude (Cosson et al., 2000; Alavi et al., 2008). Ecologically, area of spawning in relation to water flow may also influence sperm motility of different species. The fin(s) structure along the flagellum which have been shown to be present in some fish species such as turbot (Cosson et al., 2008a), sturgeons (Psenicka et al., 2008) and pike (this study) also plays an important role for wave efficiency and subsequently sperm velocity.

4.5. Osmolality effects on flagellar wave parameters during sperm motility

Most components of the axonemal fine structure are involved in flagellar beating during sperm movement (Inaba, 2003). The inner arms which are both necessary and sufficient to generate flagellar bends, determine the size and shape of the waveform; the outer dynein arms add power and increase beat frequency (Brokaw and Kamiya, 1987) and inner arm dynein plays a distinct role in generation and control of motility (Porter and Sale, 2000). The central pair of microtubules and radial spokes interact with the inner arms to control the flagellar waveform (Smith and Lefebvre, 1997). It was demonstrated that ATP induces sliding between specific subsets of doublet microtubules and that Ca^{2+} ions induce changes in waveform (Wargo et al., 2004). At the same osmolality, after activation of spermatozoa and when ATP content of sperm lessons, the wave length and amplitude decrease in sturgeons (Cosson et al., 2000; Alavi et al., 2008d). However, the

present study showed that the number of waves and curvatures differed significantly in relation to osmolality of the activation media. By increasing the osmolality of the activation media, the number of wave and curvatures along the flagellum will increase and this can be accompanied by a decrease in sperm velocity (Cosson et al., 2008a,b). This feature has been also reported in cod, sea bass and pike (Cosson et al., 2008b). Taken together, flagellar waveform depends on both several extra- (such as osmolality) and intracellular parameters (ATP content and Ca^{2+} concentration) and both of these affect the axonemal beating pattern.

Acknowledgements:

This study was supported by USB RIFCH No: MSM 6007665809, GACR No. 524/06/0817 and IAA608030801. The authors appreciate funding support received from FAPEMIG (CVZ APQ 2578-5-04/07), Brazil for Ana Viveiros. The authors warmly thank Martin Psenicka and Martina Tesarova for their helps during preparation of samples for SEM and Ivana Samkova and Marie Pcena for their technical assistants.

References

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes: (I) Effects of pH and temperature. Cell Biol. Int. 29, 101–110.

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A. 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction 128, 819–828.

Alavi, S.M.H., Cosson, J., Kazemi, R., 2006. Semen characteristics in *Acipenser persicus* in relation to sequential stripping. J. Appl. Ichthyol. 22 (Suppl. 1), 400–405.

Alavi, S.M.H., Cosson, J., Legendre, M., Rodina, M., Linhart, O., 2008a. A review on sperm motility characteristics in freshwater fish. In: *Aquaculture Europe: Resource management*, (Kamler E, Dabrowski K, eds). Krakow, September 15 – 18, Pp. 256–257.

Alavi, S.M.H., Linhart O., Coward K., Rodina M., 2008b. Fish spermatology: Implication for aquaculture management. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, R. (Eds.), Alpha Science Ltd, Oxford, UK. pp. 397–460.

Alavi, S.M.H., Psenicka, M., Policar, T., Linhart, O., 2008c. Morphology and fine structure of *Barbus barbus* (Teleostei: Cyprinidae) spermatozoa. J. Appl. Ichthyol. 24, 378–381.

Alavi, S.M.H**,** Rodina, M., Cosson, J., Psenicka, M., Linhart, O., 2008d. Roles of extracellular Ca^{2+} and pH on motility and flagellar wave form parameters in sturgeon spermatozoa. Cybium 32(2), 124–126.

Alavi, S.M.H., Rodina, M., Policar, T., Kozak, P., Psenicka, M., Linhart, O., 2007. Semen of *Perca fluviatilis* L: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology 68, 276–283.

Babiak, I., Glogowski, J., Luczynski, M.J., Kucharczyk, D., Luczynski, M., 1995. Cryopreservation of the milt of the northern pike. J. Fish Biol. 46, 819 – 828.

Babiak, I., Glogowski, J., Luczynski, M.J., Luczynski, M., 1997. Effect of individual male variability on cryopreservation of northern pike, *Esox lucius* L, sperm. Aqua. Res. 28, 191–197.

Billard, R., 1970. Ultrastructure compare de spermatozoids de quelques poissions Teleosteens. In: *Comparative Spermatology*, Baccetti, B., (Ed.). Pp. 71–79. Academic Press, New York.

Billard, R., 1983. Effects of ceolomic and seminal fluids and various saline diluents on the fertilizing ability of spermatozoa in the Rainbow trout, *Salmo gairdneri*. J. Reprod. Fertil. 68, 77–84.

Billard, R., Cosson, M.P., 1992. Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122–131.

Billard, R., Marcel, J., 1980. Stimulation of spermiation and induction of ovulation in pike (*Esox lucius*). Aquaculture 21, 181–195.

Billard, R., Cosson, J., Crim, L.W., Suquet, M., 1995a. Sperm physiology and quality. In: *Brood stock management and egg and larval quality*. Bromage, N.R., Roberts, R.J., (Eds.). Pp. 25–52. Blackwell Science. Amsterdam.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995b. Biology of sperm and artificial reproduction in carp. Aquaculture 124, 95–112.

Brokaw, C.J., Kamiya, R., 1987. Beding patterns of Chlamydomonas flagella. IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. Cell Motil. Cytoskel. 8, 68–75.

Ciereszko, A., 2008. Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity and cryopreservation success in fish. In: *Fish Spermatology,* Alavi, S.M.H., Cosson, J.J., Coward, R., Rafiee, G., (Eds.). Pp. 215–240. Alpha Science Ltd, Oxford.

Ciereszko, A., Glogowski, J., Dabrowski, K., 2000. Biochemical characteristics of seminal plasma and spermatozoa of freshwater fishes. In: *Cryopreservation of Aquatic Species*, Tiersch, T.R., Mazik, P.M., (Eds.). Pp. 20–48. World Aquaculture Society, Baton Rouge, Louisiana.

Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aqua. Int. 12, 69–85.

Cosson, J., Billard, R., Redondo-Muller, C., Cosson, M.P., 1991. *In vitro* incubation and maturation of common carp, *C. carpio*, spermatozoa, Bull. Inst. Zool. Acad. Sinica Monograph, 16, 249– 61.

Cosson, J., Groison, A-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008a. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460–486.

Cosson, J., Groison, A-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008b. Marine fish spermatozoa: racing ephemeral simmers. Reproduction 136, 277–294.

Cosson, J., Billard, R., Cibert, C., Dreanno, C., Suquet, M., 1999. Ionic factors regulating the motility of fish sperm. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 161–186. Cache Rive Press.

Cosson, J., Linhart, O., Mims, S.D., Shelton, W.L., Rodina, M., 2000. Analysis of motility parameters from paddlefish and shovelnose sturgeon spermatozoa. J. Fish Biol. 56, 1348–1367.

Gallis, J.L., Fedrigo, E., Jatteau, P., Bonpunt, E., Billard, R., 1991. Siberian sturgeon spermatozoa: Effects of dilution, pH, osmotic pressure, sodium and potassium ions on motility. In *Acipenser*, Williot, P., (Ed.). Pp. 143–151. Cemagref, Bordeaux.

Gibbons, I.R., 1981. Cilia and flagella of eukaryotes. J. Cell Biol. 91, 107s – 124s.

Hulak, M., Rodina, M., Alavi, S.M.H., Linhart, O., 2008a. Evaluation of semen and urine of pike (*Esox lucius* L.): Ionic compositions and osmolality of the seminal plasma and sperm volume, density and motility. Cybium 32(2), 189–190.

Hulak, M., Rodina, M., Linhart, O., 2008b. Characteristics of stripped and testicular Northern pike (*Esox lucius*) sperm: spermatozoa motility and velocity. Aquat. Living Resour. 21, 207–212.

Inaba, K., 2003. Molecular architecture of the sperm flagella: Molecules for motility and signaling. Zool. Sci. 20, 1043–1056.

Inaba, K., 2008. Molecular mechanisms of the activation of flagellar motility in sperm. In: *Fish Spermatology,* Alavi, S.M.H., Cosson, J.J., Coward, R., Rafiee, G., (Eds.). Pp. 267–279. Alpha Science Ltd, Oxford.

Ishijima, S., Hara, M., Okiyama, M., 1998. Comparative studies on spermatozoan motility of Japanese fishes. Bull. Ocean Res. Inst. Univ. Tokyo 33, 139– 52.

Jamieson, B.G.M., 1991. *Fish Evolution and Systematics: Evidence from Spermatozoa*. Cambridge University Press, Cambridge. ISBN 0 521 41304 4.

Koldras, M., Moczarski, M., 1983. Properties of pike *Esox lucius* L. milt and its cryopreservation. Pol. Arch. Hydrobiol. 30, 69–78.

Kouril, J., Hamackova, J., 1975. Fecundity of pond-reared pike (*Esox lucius* L). Czech J. Anim. Sci. 20, 841–849.

Krasznai, Z., Marian, T., Izumi, H., Damjanovich, S., Balkay, L., Tron, L., Morisawa, M., 2000. Membrane hyperpolarization removes inactivation of Ca^{2+} channels leading to Ca^{2+} influx and initiation of sperm motility in the common carp. Proc. Nat. Acad. Sci. 97, 2052–2067.

Lahnsteiner, F., Patzner, R.A., 2008. Sperm morphology and ultrastructure in fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, R., Rafiee, G., (Eds.). Pp. 1–61. Alpha Science Ltd, Oxford.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R., 1995. Fine structure and motility of spermatozoa and composition of the seminal plasma in the perch. J. Fish Biol. 47, 492–508.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R., 1997. Sperm structure and motility of freshwater teleost Cottus gobio. J. Fish Biol. 50, 564–574.

Linhart, O., 1984. Evaluation of pike and European catfish sperm. Bulletin VURH Vodnany 20, 22–33.

Linhart, O., Alavi, S.M.H., Rodina, M., Gela, D., Cosson, J., 2008. Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (*Cyprinus carpio*). J. Appl. Ichthyol. 24, 386–396.

Linhart, O., Mims, S.D., Shelton, W.L., 1995. Motility of spermatozoa from Shovelnose sturgeon, *Scaphirhynchus platorynchus*, and Paddlefish, *Polyodon spathula*. J. Fish Biol. 47, 902 – 909.

Linhart, O., Rodina, M., Gela, D., Kocour, M., 2004. Optimization of artificial propagation in European catfish, *Silurus glanis* L. Aquaculture 235, 619 – 632.

Linhart, O., Slechta, V., Slavik, A., 1991. Fish sperm composition and biochemistry. Bull Inst Zool, Acad. Sin. 16, 285–311.

Lin. F., Dabrowski, K., 1996. Characteristics of muskellunge spermatozoa. I: Effects of ions and osmolality on sperm motility. Trans. Am. Fish. Soci. 125, 187–202.

Lin, F., Liu, L., Dabrowski, K., 1996. Characteristics of muskellunge spermatozoa. I: Ultrastructure of spermatozoa and biochemical composition of semen. Trans. Am. Fish Soci. 125, 187–194.

Mattei, X., 1991. Spermatozoon ultrastructure and its systematic implications in fishes. Can. J. Zool. 69, 3038–3055.

Morisawa, M., 1985. Initiation mechanism of sperm motility at spawning in teleosts. Zool. Sci. 2, 605–615.

Morisawa, S., Morisawa, M., 1986. Acquisition of potential for sperm motility in rainbow trout and chum salmon. J. Exp. Biol. 126, 89–96.

Morisawa, S., Morisawa, M., 1988. Induction of potential for sperm motility by bicarbonate and pH in rainbow trout and chum salmon. J. Exp. Biol. 136, 13–22.

Morisawa, M., Yoshida, M., 2005. Activation of motility and chemotaxis in the spermatozoa: From invertebrates to humans. Reprod. Med. Biol. 4, 101–114.

Morisawa, M., Oda, S., Yoshida M., Takai, H., 1999. Transmembrane signal transduction for the regulation of sperm motility in fishes and ascidians. In: *The male gamete: From basic to clinical applications*, Gagnon, C. (Ed.). Pp. 149–160. Cache Rive Press.

Morisawa, M., Suzuki, K., Morisawa, S., 1983a. Effects of potassium and osmolality on spermatozoan motility of salmonid fishes. J. Exp. Biol. 107, 105–113.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., Yasuda, K., 1983b. Effect of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. J. Exp. Zool. 107, 95–103.

Perchec-Poupard, G., Paxion, C., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1998. Initiation of carp spermatozoa motility and early ATP reduction after milt contamination by urine. Aquaculture 160, 317–328.

Porter, M.E., Sale, W.S., 2000. The 9+2 axoneme anchors multiple inner arm dyneins and a network of kinase and phosphatase that control motility. J. Cell Biol. 151, F37–F42.

Psenicka, M., Alavi, S.M.H., Rodina, M., Cicova, Z., Gela, D., Cosson, J., Nebesarova, J., Linhart, O., 2008. Morphology, chemical contents and physiology of chondrostean fish sperm: A comparative study between Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*). J. Appl. Ichthyol. 24, 371–377.

Psenicka, M., Rodina, M., Nebesarova, J., Linhart, O. 2006. Ultrastructure of spermatozoa of tench (*Tinca tinca*) observed by means of scanning and transmission electron microscopy. Theriogenology 66, 1355–1363.

Rodina, M., Gela, D., Kocour, M., Alavi, S.M.H., Hulak, M., Linhart, O., 2007. Cryopreservation of tench, *Tinca tinca*, sperm: Sperm motility and hatching success of embryos. Theriogenology 67, 931–940.

Smith, E.F., Lefebvre. P.A., 1997. The role of central apparatus components in flagellar motility and microtubule assembly. Cell Motil. Cytoskel. 38, 1–8.

Stein, H., 1981. Licht- und elektronenoptische untersuchungen an den spermatozoen verschiedener susswasserknochenfische (Teleostei). Zeitschrift fur Angewandte Zoologie 68, 183–198.

Steinberg, D., 1992. Northern Pike and Muskie. CY DeCrosse Inc. page 20.

Stoss, J., 1983. Fish gamete preservation and spermatozoan physiology. In *Fish Physiology IXB*, Hoar, W.S., Randall, D.J., Donaldson, E.M., (Eds.). Pp. 305–350. Academic Press, New York.

Toth, G.P., Ciereszko, A., Christ, S.A., Dabrowski, K., 1997. Objective analysis of sperm motility in the Lake sturgeon (*Acipenser fulvescens*): Activation and inhibition conditions. Aquaculture 154, 337–348.

Wargo, M.J., McPeek, M.A., Smith, E.F., 2004. Analysis of microtubule sliding patterns in Chlamydomonas flagellar axonemes reveals dynein activity on specific doublet microtubules. J. Cell Sci. 117, 2533–2544.

Chapter 5

Biology of sperm in *Acipenser ruthenus* (Chondrostei; Acipenseridae)

Article 7: **Alavi, S.M.H.,** Rodina, M., Cosson, J., Psenicka, M., Linhart, O., 2008. Roles of extracellular Ca2+ and pH on motility and flagellar wave form parameters in sturgeon spermatozoa. Cybium. 32(2), 124–126.

Article 8: **Alavi, S.M.H.,** Manaskova-Postlerova, P., Koubek, P., Hatef. A., Cosson, J., Rodina, M., Konno, A., Inaba, K., Peknicova, J., Gela, D., Linhart, O., Ciereszko, A., Trypsin and Chymotrypsin – Like Activities in Sturgeon Sperm and Their Effects on Sperm Motility. In preparation

Roles of extracellular Ca^{2+} and pH on motility and flagellar waveform parameters in sturgeon spermatozoa

S. M. Hadi Alavi[1], Marek Rodina[1], Jacky Cosson[2], Martin Psenicka[1], Otomar Linhart[1]

1- University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany 389 25, Czech Republic.
2- UMR 7009 CNRS, Université Pierre et Marie Curie, 06230 Villefranche-sur-Mer, France.

Abstract

Ca^{2+} had no effect on symmetric flagellar behavior at pH 8.0. Wavelength and amplitude decreased significantly as a function of time post sperm activation, but number of curvature and waves increased. High concentrations of Ca^{2+} (2.5 mM) at high alkalinity (pH > 10.0) induced both acrosome reaction and asymmetric flagellar behavior.

Key words: Asymmetry, Acrosome reaction, Flagella

Introduction

Sturgeon spermatozoa like that of other teleosts fish are immotile in the seminal plasma due to high concentrations of K+ ions (Alavi *et al.*, 2004). Motility of sperm is induced by hypo-osmotic pressure in sturgeons as in other freshwater fishes (Alavi and Cosson, 2006). Literature revealed that the alkaline conditions of diluents enhance the motility parameters of sturgeon sperm (Alavi and Cosson, 2005). Ca^{2+} at 100 μM could reverse the K^+ inhibitory effect, but EGTA could abolish the Ca^{2+} effect (Cosson *et al.*, 2000). Cherr and Clark (1984) reported that the high concentrations of Ca^{2+} together with high pH induce the acrosome reaction in Acipenser transmontanus. The aim of the present study was to investigate the effects of Ca^{2+} and pH on sperm behavior and flagellar pattern in sterlet (*A. ruthenus*).

Methods

Sperm of three males (5 to 7 year old) with average weight of 2-5 kg were separately collected by catheter. The males were stripped 36-48 h after hormonal treatment (a single intramuscular injection of carp pituitary homogenized extract with dosage 4 mg kg^{-1} b.w). Sperm was stored on ice (0-4°C) during analysis of motility. The roles of Ca^{2+} ($CaCl_2$ from 0.5 to 10.0 mM) and pH (8.0, 9.5, 10.0 and 10.5 in 10 to 30 mM Tris-HCl) on sperm swimming patterns were tested. Buffered distilled water containing 10 mM Tris-HCl adjusted to pH 8.0 was used as control. To avoid sperm sticking to the slide, 0.1 % BSA was added. Sperm motility was activated in swimming medium at a dilution ratio 1:50. Sperm motility was recorded right after activation using a 3 CCD video camera mounted on a dark-field microscope under stroboscopic light. Sperm motility (velocity and percentage of motile cells) and flagellar waveform (wave length, wave amplitude, number of curvatures and number of waves) parameters were

analyzed on successive video frames by a micro image analyzer according to Cosson *et al.* (2000). Statistical significance ($P < 0.05$) was tested using analysis of variance (ANOVA, SPSS 10.0).

Results and discussion

The effects of Ca2+ on sperm motility, velocity and flagellar parameters and behavior at pH 8.0

The percentage of motile sperm was measured 100% at 15 s post sperm activation. No significant difference was observed between buffered distilled water and different concentrations of Ca^{2+} in terms of the percentage of motile sperm at 15 s post activation ($P > 0.05$). At 120 s post sperm activation, the percentage of motility reached 33.00, 65.02, 65.22 and 72.92 % after activation in buffered distilled water and 0.5, 1.0 and 2.5 mM Ca^{2+} concentrations, respectively. Sperm velocity increased when the Ca^{2+} concentration was increased in swimming medium. The highest sperm velocity was observed at 2.5 mM Ca^{2+} and the lowest was observed after activation of sperm in buffered distilled water or 0.5 mM Ca^{2+} (Fig. 1). Alavi *et al.* (2004) reported a high sensivity of *A. persicus* sperm to concentrations of Ca2+ ions when exceeded 3 mM in swimming medium. During the progress of the motility period, the wave pattern of sturgeon spermatozoa rapidly evolves from a fully beating situation to a partially beating situation. The same wave pattern has been reported in paddlefish and shovelnose sturgeon (Cosson *et al.*, 2000). At pH 8.0 and different concentrations of Ca^{2+}, spermatozoa showed symmetric flagellar pattern and Ca^{2+} did not affect the flagellar behavior (Fig. 2).

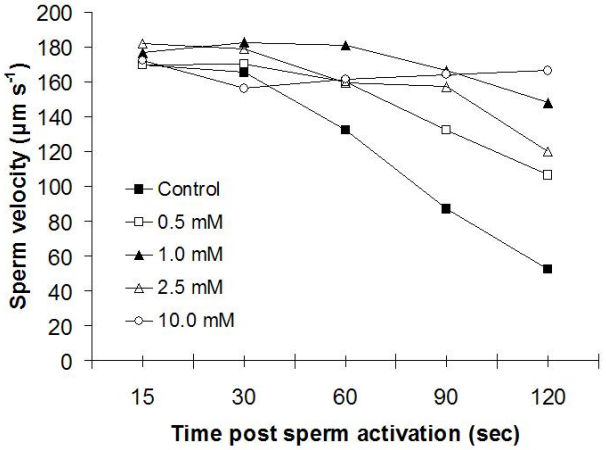

Fig. 1: Sperm velocity in *A. ruthenus* after activation in media of different concentrations of Ca^{2+} at pH 8.0.

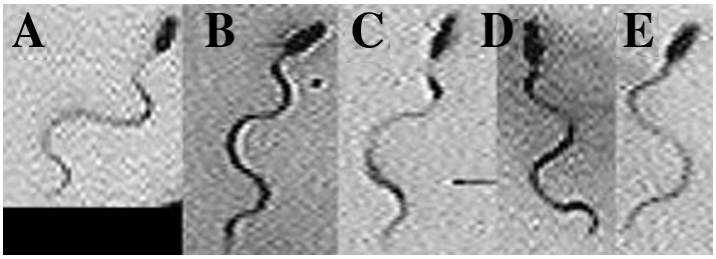

Fig. 2: Flagellar wave forms of *A. ruthenus* spermatozoa at 15 s post activation in different concentrations of Ca^{2+} (**B**: 0.5, **C**: 1.0, **D**: 2.5, and **E**: 10.0 mM) at pH 8.0. Distilled water containing 10 mM Tris-HCl and pH adjusted to 8.0 was used as control (**A**).

Flagellar parameters of sperm at different concentrations of Ca^{2+} adjusted to pH 8.0 are shown in figure 3. The wave length decreased significantly as a function of time post sperm activation. At 15 s post sperm activation, the lowest (13.11 ± 2.21 µm) and highest (15.54 ± 3.34 µm) wave length were observed after activation of sperm in swimming medium containing 0.5 mM Ca^{2+} and buffered distilled water, respectively ($P < 0.05$). Wave amplitude also showed a significant decrease during the motility period. But, no significant differences were observed among buffered distilled water and different concentrations of Ca^{2+} in terms of wave amplitude at 15 s post sperm activation ($P > 0.05$). Number of curvature increased during time post sperm activation. The lowest (3.93 ± 0.80) was observed after activation of sperm in buffered distilled water ($P < 0.05$). The number of waves also increased during motility phase of spermatozoa. Both the highest number of waves (2.67 ± 0.87) and number of curvature (6.00 ± 1.32) were observed after activation of sperm in swimming medium containing 0.5 mM Ca^{2+}. Cosson *et al.* (2000) reported that the head trajectories remain close to linearity during the entire period after activation. Furthermore, the Ca^{2+} has no major effect on motility, reflecting the absence of asymmetric beating.

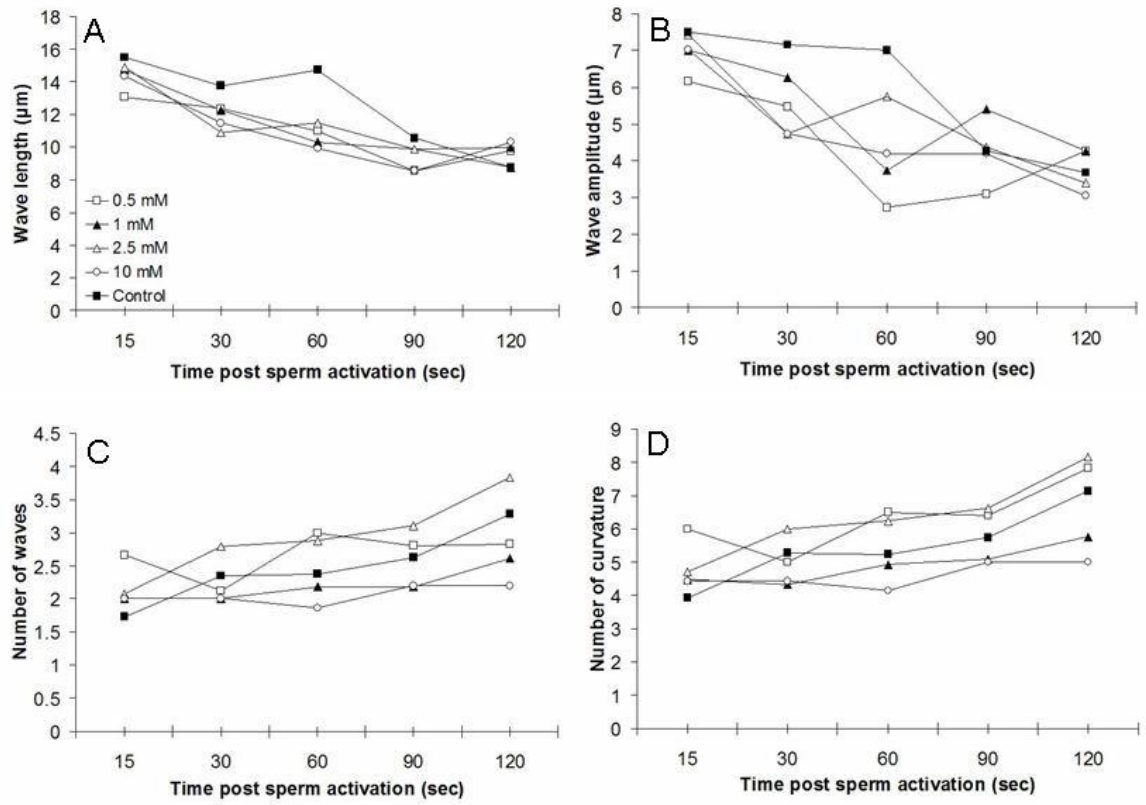

Fig. 3: Effects of Ca^{2+} on wave length (**A**), wave amplitude (**B**), number of waves (**C**), number of curvature (**D**) of *A. ruthenus* spermatozoa at pH 8.0.

Effects of Ca2+ on sperm motility, velocity and flagellar behavior at different pH

The percentage of motile sperm was measured 100% at the different concentrations of Ca^{2+} tested. But, the velocity of sperm dropped 0.00 (μm s^{-1}) after activation of sperm in 0.5 and 1.0 mM Ca^{2+} when the pH was adjusted to ≥ 10, and after activation of sperm in 2.5 and 10.0 mM Ca^{2+} when the pH was adjusted to ≥ 9.5 at 15 s post sperm activation (Fig. 4). It was due to sticking of sperm head to the glass slide. We observed the activated sperm samples under phase contrast microscopy at 100X magnification to demonstrate if the sticking of sperm was due to reactivation of acrosome process. The results are presented in figure 5. On the other hand, high concentrations of Ca^{2+} at high alkalinity of swimming medium can induce acrosome reaction in sturgeon spermatozoa. Cherr and Clark (1984) reported that Ca^{2+} is necessary for inducing acrosome reaction of A. transmontanus spermatozoa. In addition, the asymmetric behavior of flagella was observed in the same condition, meaning high concentrations of Ca^{2+} and pH. Figure 6 shows the asymmetric behavior of flagella after activation at different pH at 2.5 mM Ca^{2+}. The flagellar beating changes from symmetric observed at pH 8.0 to asymmetric at pH 9.5, 10.0 and 10.5. The role of Ca^{2+} in regulating the degree of asymmetry of the flagellar bending waves has been reported in sea urchin (Gibbons, 1982) and rainbow trout (Cosson *et al.*, 1991). To our knowledge, this is the first report demonstrating the asymmetric behavior of sturgeon spermatozoa after activation in the presence of Ca^{2+} at high alkalinity condition.

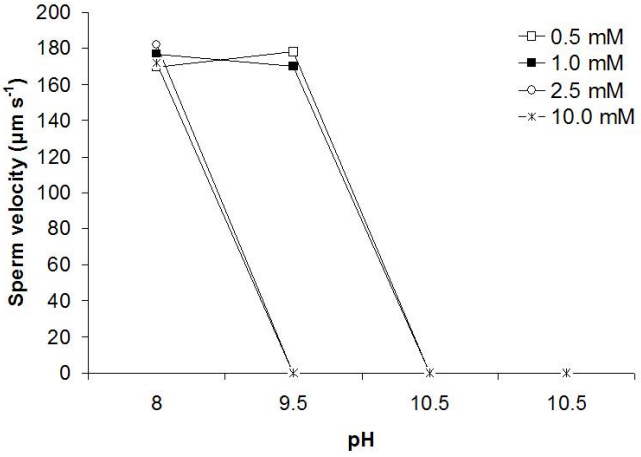

Fig. 4: Sperm velocity in *A. ruthenus* after activation in different concentrations of Ca^{2+} and at different pH.

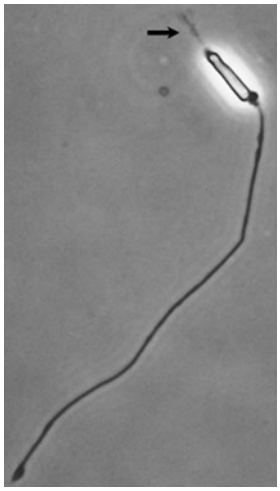

Fig. 5: Acrosome reaction in *A. ruthenus* spermatozoa after activation of sperm in 2.5 mM Ca^{2+} at pH 10.0.

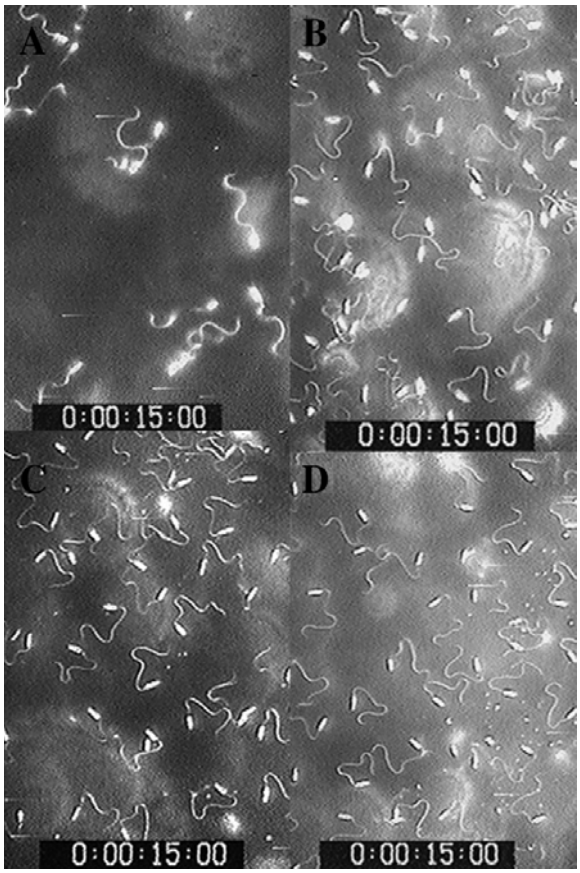

Fig. 6: Symmetric and asymmetric pattern of flagellar movement in *A. ruthenus* at 15 s post sperm activation. Motility was triggered in 2.5 mM Ca^{2+} at pH 8.0 (**A**), 9.5 (**B**), 10.0 (**C**) and 10.5 (**D**).

References

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes: (I) Effects of pH and temperature. Cell Biol. Int. 29, 101–110.

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A., 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction 128, 819–828.

Cherr, G.N., Clark, W.N., 1984. Acrosome reactions in sperm from the white sturgeon, *Acipenser transmontanus.* J. Exp. Zool. 232, 129–139.

Cosson, J., Linhart, O., Mims, S.D., Shelton, W., Rodina, M., 2000. Analysis of motility parameters from paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) spermatozoa. J. Fish Biol. 56, 1348–1367.

Cosson, M.P., Cosson, J., Billard, R., 1991. Synchronus triggering of trout sperm is followed by an invariable set sequence of movement parameters whatever the incubation medium. Cell Motil. Cytoskel. 20, 55–68.

Gibbons, B.H., 1982. Effects of organic solvents on flagellar asymmetry and quiescence in sea urchin sperm. J. Cell Sci. 54, 115–135.

Trypsin and Chymotrypsin – Like Activities in Sturgeon Sperm and Their Effects on Sperm Motility

S. M. Hadi Alavi[1], Pavla Manaskova-Postlerova[2], Pavel Koubek[2], Azadeh Hatef[1], Jacky Cosson[3], Marek Rodina[1], Aru Konno[4], Kazuo Inaba[4], Jana Peknicova[2], David Gela[1], Otomar Linhart[1], Andrzej Ciereszko[5]

1- Research Institute of Fish Culture and Hydrobiology, University of South Bohemia, Vodnany 389 25, Czech Republic
2- Lab. Diagnostics for Reproductive Medicine, Institute of Biotechnology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague, Czech Republic
3- UMR 7009 CNRS, UPMC, 06230 Villefranche sur mer, France
4- Shimoda Marine Research center, University of Tsukuba, Shimoda 5-10-1, Shizuoka 415-0025, Japan
5- Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn 10-747, Poland.

Abstract

Sturgeon spermatozoa have acrosome, but its real function is not well known. In this study, protease activities was determined in sperm of sterlet (*Acipenser ruthenus*) and the effects of trypsin- (AGB) and chymotrypsin-like (TPCK) inhibitors on sperm motility were investigated. The results showed that both trypsin- (AGB) and chymotrypsin- like (TPCK) inhibitors had significant effect on sperm motility in dose and time dependent manner (ANOVA, $P < 0.001$). Percentage of motile spermatozoa significantly decreased when AGB or TPCK was added to activation medium >0.25 mM or >0.5 mM. TPCK inhibitory effect was more effective than AGB at 120 sec post activation. Sperm velocity was significantly higher when AGB concentrations was higher than 0.25 mM compared to those of controls as well low concentrations of AGB (0.0625 and 0.125 mM). In presence of TPCK at 1mM, significantly the highest sperm velocity was observed compared to controls and lower concentrations of TPCK. In general point of view, low concentrations of both TPCK (0.0625 – 0.5 mM) and AGB (0.0625 – 0.25 mM) lead to lower sperm velocity than controls. The protease activity was significantly higher in sperm extract than seminal plasma. In samples of sperm acidic extract, total protease and serine activities were accumulated in protein band with relative molecular mass of 33kDa, which seems to be an acrosin. We conclude that (1) protease seems to have a regulatory function of sperm motility in sturgeon. (2) Protease activities differ among sturgeon species. (3) Further studies are needed to understand if sturgeon acrosin- like activity shares many properties with mammalian acrosin and its role during fertilization.

Key words: AGB, TPCK, Sperm motility and velocity, Acrosin

1. Introduction

It has been already demonstrated that there is family-, species- and subspecies-specific differences in terms of the fine structure of spermatozoa with functionality

(motility and fertility) (Jamieson, 1991; Lahnsteiner and Patzner, 2008) and physiological and biochemical characteristics of spermatozoa (Ciereszko, 2008). Fish sperm is usually differentiated into a head, a midpiece a flagellum with the typical 9+2 pairs of microtubules (Jamieson, 1991; Lahnsteiner and Patzner, 2008). The sturgeon spermatozoa differ from those of teleosts spermatozoa because of having an acrosome (Cherr and Clark 1984, 1985; DiLauro et al., 1998; Psenicka et al., 2007, 2008), a longer motility period (Billard et al., 1991; Cosson et al., 2000; Alavi et al., 2004) and possessing trypsin- and chymotrypsin- like activities (Ciereszko et al., 1994, 1996, 2000). It has been already clarified that acrosome of sturgeon spermatozoa is active and acrosome reaction can be induced, which is a Ca^{2+} and/or Mg^{2+} dependent event (Dettlaff et al., 1993; Ciereszko et al., 2000; Alavi et al., 2008). Acrosomal reaction can be also triggered by a 66 KDa glycoprotein, a component in egg envelope (Cherr and Clark, 1985). Nevertheless, it is not well known why despite the presence of multiple micropyles in eggs of sturgeons, spermatozoa possess an acrosome and subsequently what is the main function of acrosome in fertilization mechanism? In most teleosts, spermatozoa penetrate into the eggs through a micropyle, a canal in the egg envelops, which allows sperm direct access to the oolemma (Kudo, 1991).

The acrosome of invertebrate and vertebrate sperm possesses unique enzymatic features which enable contact with the egg and subsequently penetration. In mammals, acrosomal proteinase play main role to digest pathway for the sperm through the zona pellucida (Polakoski et al., 1973; Jelinkova et al., 2003). A trypsin- like protease (acrosin) is one of the most characteristic acrosomal enzymes which involved in fertilization (Zaneveld et al., 1973, 1982). In contrast to teleost sperm with no acrosin activity and even anti- proteinase (including anti-acrosin) activity (Dabrowski and Ciereszko, 1994; Kowalski et al., 2003; Wojtczak et al., 2004), sturgeon spermatozoa have acrosin- like activity (Ciereszko et al., 1996, 2000). Ciereszko et al., (1994) documented amidase activity in lake sturgeon (*Acipenser fulvescens*) and white sturgeon (*A. transmontanus*), which was inhibited by benzamidine and soybean trypsin inhibitor (SBTI) and by N-α-p-tosyl-L-lysine chloromethyl ketone-HCl (TLCK), but not by N-tosyl-L-phenylalanine chloromethyl keton (TPCK), indicating the presence of trypsin-like activity in sturgeon spermatozoa. Although acrosin-like activity shares many characteristics of mammalian acrosin, but species- specific was already shown in sturgeon spermatozoa such as inhibition of acrosin- like activity by Triton X-100 and catalytic efficiency at low temperatures (Ciereszko et al., 1994, 1996). Nevertheless, it is not well documented that if protease activities differ among different species of sturgeons?

High potassium concentration in seminal plasma inhibits the motility of sturgeon spermatozoa (Gallis et al., 1991; Toth et al., 1997; Alavi et al., 2004). A hypo-osmotic shock is necessary for triggering of sperm motility in sturgeons (Linhart et al., 1995; Cosson and Linhart, 1996; Cosson et al., 2000; Alavi et al., 2004) and the motility is also affected by osmolality of activation media and completely inhibited at osmolality >80 mOsmol kg^{-1} depending on species (Gallis et al., 1991; Linhart et al., 1995; Alavi et al., 2004; Lahnsteiner et al., 2004). A few studies have claimed roles of proteases in regulation of sperm motility. In se urchin, fish and mammalian spermatozoa, some protease inhibitors (trypsin or chymotrypsin-like inhibitors) block the motility of intact or demembranated reactivated spermatozoa (de Lamirande et al., 1983; de Lamirande and Gagnon, 1986; Gagnon and Cosson, 1987; Cosson and Gagnon, 1988) through changing

of signal transduction in cAMP-dependent regulation of sperm motility (Inaba et al., 1998).

For the first time in present study, we investigated the effects of trypsin- and chymotrypsin- like inhibitors on motility of sterlet (*Acipenser ruthenus*) spermatozoa. Protease activities were also measured in sperm suspension and seminal plasma and a preliminary study was done to find acrosin from sturgeon semen using SDS-PAGE.

2. Materials and methods

2.1 Broodfish and gamete collection

Sperm of 6 males (5 to 7 year old) with average weight of 2 - 5 kg were used as experimental material. Broodfish were transferred from pond to a broodfish recirculated tank (4000 liter; temperature 13°C, flow rate 0.2 l s^{-1}) in hatchery 5 days before spawning induction by a single intramuscular injection of carp pituitary homogenized extract with dosage 4 mg kg^{-1} b.w. Sperm was collected 48 hours after hormonal injection by catetherisation from urogenital papilla of each male separately and collected in 250 ml cell culture containers.

2.2. Chemicals

N-α-p-tosyl-L-lysine chloromethyl ketone-HCl (TLCK), N-tosyl-L-phenylalanine chloromethyl keton (TPCK), a'-acetamidophenyl 4-guanidinobenzoate (AGB), Dimethyl sulfoxide (DMSO), Teleostean gelatin, EDTA, benzamidine hydrochloride, *N*-benzoyl-dl-arginine-4-nitroanilide.HCl (dl-BAPA) and Triton X-100 were purchased from Fluka Buch, Switzerland or Sigma-Aldrich, St Louis, MO or Serva, Heidelberg, Germany.

2.3. Effects of protease inhibitors on sperm motility

The effects of TLCK, TPCK and AGB were tested on sperm motility parameters both percentage of motile spermatozoa and sperm velocity at different concentration (1.0, 0.5, 0.25, 0.125 and 0.0625 mM). The protease inhibitors were dissolved in 10 mM NaCl, 10 mM Tris – HCl, pH 8.5 containing 1% DMSO. To avoid sperm sticking to the slide, 0.1% BSA was added. Two activation media were used as control: (a) 10 mM NaCl, 1% DMSO, 10 mM Tris – HCl, pH 8.5 and 10 mM NaCl, 10 mM Tris – HCl, pH 8.5. Analysis of sperm motility was carried out in duplicate for each male at each concentration of protease inhibitor.

2.4. Sperm motility analysis

Sperm velocity (μm s^{-1}) and percentage of motile spermatozoa (%) were measured after triggering sperm motility in activation medium under dark-field microscopy (NIKON Optiphot 2, Tokyo, Japan) (20x objective magnification). Sperm motility was recorded with a three CCD video camera (SONY DXC-970MD, Tokyo, Japan) mounted on the microscope. The successive positions of the head of spermatozoa were measured from five frames using a video-recorder (SONY SVHS, SVO-9500 MDP,

Tokyo, Japan) and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows).

2.5. Extract of acrosomal proteins

The acrosomal content of spermatozoa was isolated as the sperm acidic extract. We applied a modified procedure described by Cechova et al., (1988) used for the isolation of proacrosin of boar spermatozoa. Suspension of washed spermatozoa (10^8 cells/ml in PBS) were incubated with solution (1:1) containing 2% acetic acid, 50 mM NaCl, 50 mM benzamidine hydrochloride and 10 mM EDTA overnight on ice. After centrifugation (21 000 g, 30 min), supernatants were desalted on Protein Desalting Spin Columns (Pierce, Rockford, IL) and lyophilized.

2.6. Protease activity measurement

The protease activity was determined by the photometric method using *N*-benzoyl-dl-arginine-4-nitroanilide.HCl (dl-BAPA) solution as a substrate in 0.2 M Tris-HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$. The protease activity determined as amidase activity after hydrolysis of dl-BAPA was monitored by absorbance measurements at 405 nm (Cechova et al., 1994).

2.7. SDS electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% slab gel. The protein samples (acrosomal extracts) were dissolved in non-reducing buffer and boiled for 2.5 min at 100°C. The molecular masses of the separated proteins were estimated using prestained precision protein standards All Blue from Bio-Rad (Hercules, CA) run in parallel.

2.8. Substrate zymography

For determination of proteinases, the gelatin-substrate gel electrophoresis was used (Siegel and Polakoski, 1985). Teleostean gelatin was added to 10% SDS-PAGE at a final concentration of 0.2%. After electrophoresis, the gels were rinsed in 2.5% Triton X-100 (Serva, Heidelberg, Germany) in 50 mM Tris-HCl, pH 8.4 with 5 mM $CaCl_2$ for 1 hr to remove SDS and then transferred into the assay buffer (50 mM Tris-HCl, pH 8.4 with 5 mM $CaCl_2$) for determination of total proteolytic activity. For detection of serine proteinase only, 5mM EDTA (Sigma-Aldrich, St. Loise, MO) were added to buffer. Gels were incubated at 37°C overnight and then stained in the Coomassie Brilliant Blue (Serva, Heidelberg, Germany).

2.9. Data analysis

The results are shown as mean ± standard deviation (S.D.). Statistical significance was tested using analysis of variance (ANOVA, SPSS 10.0). Probability values < 0.05 were considered significant.

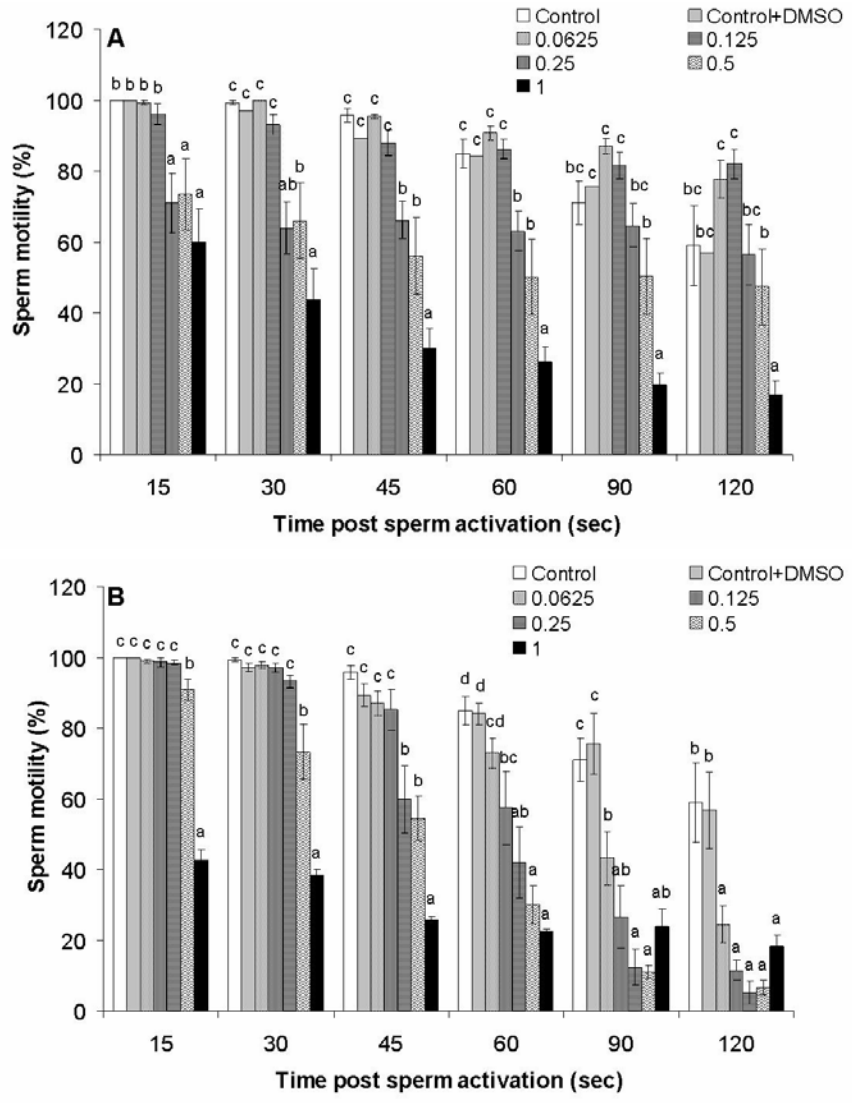

Fig. 1: Effects of trypsin- like inhibitor (AGB) and chymotrypsin- like inhibitor (TPCK) on the percentage of motile spermatozoa in *Acipenser ruthenus*. AGB and TPCK were dissolved in 10 mM NaCl, 10 mM Tris – HCl, pH 8.5 containing 1% DMSO. Control medium is containing 10 mM NaCl, 10 mM Tris – HCl, pH 8.5. 1% DMSO is added to control as defined Control + DMSO. At the same time, values with the same letters are not significantly different ($P > 0.05$, ANOVA). Analysis of sperm motility was carried out in duplicate for each male at each concentration of protease inhibitor (n=3).

3. Results

3.1. Effects of protease inhibitors on sperm motility

TLCK did not show significant effect on sperm motility (data is not shown). The results showed that both trypsin- (AGB) and chymotrypsin- like (TPCK) inhibitors had significant effect on sperm motility, which was concentration (dose) and time dependent

(ANOVA, $P < 0.001$). Percentage of motile spermatozoa significantly decreased when AGB was added to activation medium more than 0.25 mM (Fig. 1a). AGB had no or only marginal effects on motility at concentrations 0.0625 and 0.125 mM. TPCK had almost the same inhibitory effect, but not at 0.25 mM. TPCK more than 0.5 mM showed significant inhibitory effect on sperm motility (Fig 1b). Nevertheless, TPCK inhibitory effect was more effective than AGB at 120 sec post activation (Fig. 1a,b). The inhibitory effect of AGB was almost constant during sperm activation except of adding 1 mM in activation media, while in case of TPCK the percentage of motile spermatozoa significantly decreased compared to controls at 60 sec post activation.

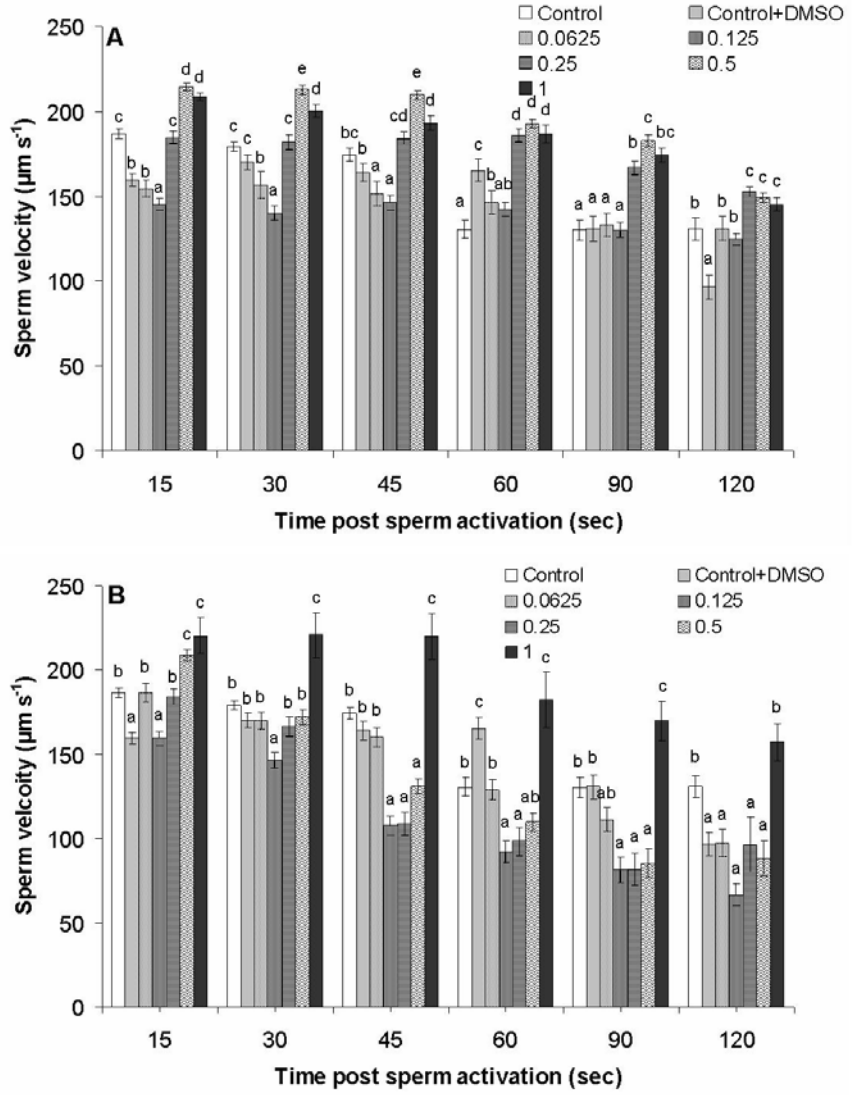

Fig. 2: Effects of trypsin-like inhibitor (AGB) and chymotrypsin- like inhibitor (TPCK) on sperm velocity in *Acipenser ruthenus*. AGB and TPCK were dissolved in 10 mM NaCl, 10 mM Tris – HCl, pH 8.5 containing 1% DMSO. Control medium is containing 10 mM NaCl, 10 mM Tris – HCl, pH 8.5. 1% DMSO is added to control as defined Control + DMSO. At the same time, values with the same letters are not significantly different ($P > 0.05$, ANOVA). Analysis of sperm motility was carried out in duplicate for each male at each concentration of protease inhibitor (n=3).

Sperm velocity decreased in all treatments as a function of time (Fig. 2). Sperm velocity was significantly higher when AGB concentrations was higher than 0.25 mM compared to those of controls as well low concentrations of AGB (0.0625 and 0.125 mM) (Fig. 2a). From time of triggering of sperm motility to 120 sec post activation, significantly the highest sperm velocity was observed at 1 mM concentration of TPCK compared to controls and lower concentrations of TPCK (Fig. 2b). In general point of view, low concentrations of both TPCK (0.0625 – 0.5 mM) and AGB (0.0625 – 0.25 mM) lead to lower sperm velocity than controls.

3.2. Proteinase activities in semen (sperm cells and seminal plasma)

Figure 3 shows the protease activity determined as amidase activity after hydrolysis. Significantly higher amidase activity was observed in acidic extract (2% acetic acid + 50 mM NaCl) than seminal plasma. Figure 4 shows total protease and serine protease activities in sperm acidic extract and seminal plasma by gelatin substrate zymography. In seminal plasma samples (SP1-3), protease activity was observed in protein bands with high molecular masses. On the other hand, in samples of sperm acidic extract (S1-3) total protease and serine protease activities were accumulated in protein band with relative molecular mass of 33kDa. Figure 5 shows our preliminary results that protein band with 33kDa with serine activity is acrosin.

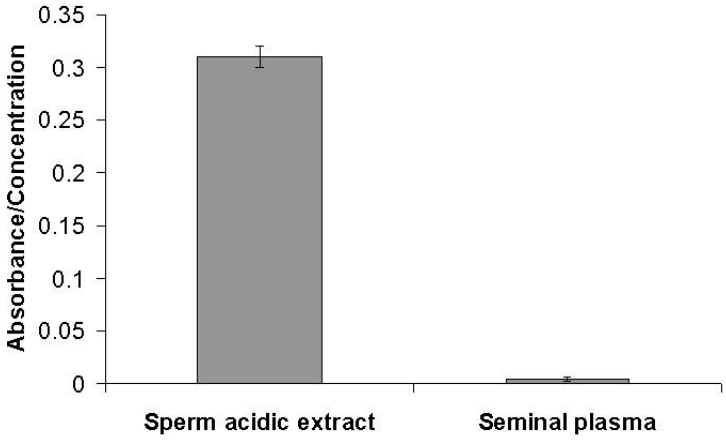

Fig. 3: Significant difference of protease activity between sperm extract and seminal plasma of *Acipenser ruthenus* by photometric method. The protease activity determined as amidase activity after hydrolysis of dl-BAPA monitored by absorbance measurements at 405 nm. (n=3)

4. Discussion

Since the measurement of protease activities in sturgeon sperm, great attention was given to study effects of protease inhibitors on sperm motility, purify and localize protease in semen (spermatozoa cells and seminal plasma) and to understand their roles in acrosome reaction and fertilization. In present study, we (a) investigated effects of trypsin- and chymotrypsin- like inhibitors on sperm motility, (b) measured protease activities in sperm suspension and seminal plasma and (c) isolate acrosin from sturgeon sperm.

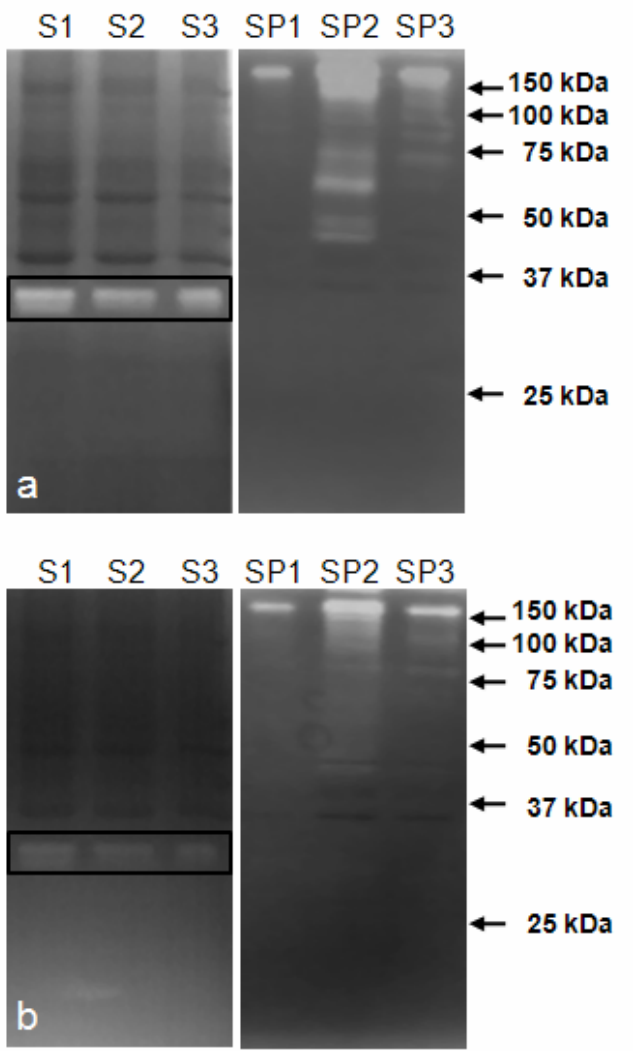

Fig. 4: Total protease (a) and serine protease (b) activities in sperm extract and seminal plasma of 3 different samples of sperm of *Acipenser ruthenus* by substrate zymography. S1-3 – samples of sperm acidic extract, SP1-3 – samples of seminal plasma.

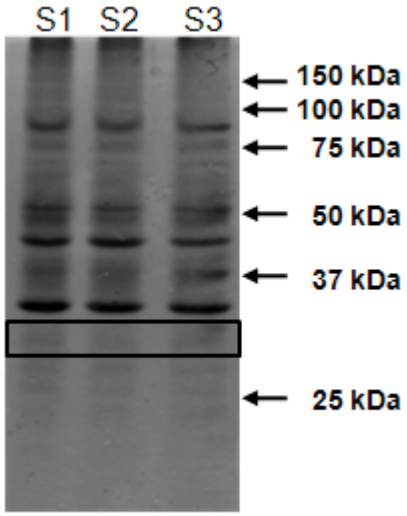

Fig. 5: 15% SDS-PAGE of sperm acidic extracts of 3 different samples of sperm of *Acipenser ruthenus*. It is supposed that protein band with 33kDa with serine activity is acrosin.

It was well known that some protease inhibitors block the acrosome reaction or fertilization, from invertebrate (such as tunicate or se urchin) to vertebrates (fish and mammals) (Hoshi et al., 1981; Green and Summers, 1982). In addition, Gagnon and co-workers were the first describing the significance of protease in flagellar motility of sperm. de Lamirande et al., (1983) found that a trypsin-like inhibitor, aprotinin, blocked the motility of demembranated and reactivated mammalian spermatozoa. Then, de Lamirande and Gagnon (1986) explored that some protease inhibitors and substrates with lys or arg ester bonds (such as BLT, chromozym TH and BAPNA) inhibit the motility of both demembranated reactivated and intact mammalian spermatozoa. Cosson and Gagnon (1988) and Gagon and Cosson (1987) found that the same protease inhibitors or substrates were active or inactive on mammalian spermatozoa were with or without effects, respectively, on vertebrate (common carp, *Cyprinus carpio*) and invertebrate (sea urchin, *Paracentrotus lividus*) spermatozoa. (Aprotinin and BLT blocked the reinitiating of demembranated and reactivated sperm, but soybean trypsin inhibitor and benzamidin had no effect on sperm motility.) These finding suggested that the same mechanism of motility regulation might have been preserved throughout evolution from sea urchin to mammals. However, it seems that high concentrations of protease inhibitors required for giving complete inhibition of sperm motility in sea urchin and carp compared to mammalian spermatozoa (Gagon and Cosson, 1987; Cosson and Gagnon, 1988). Further studies showed that protease inhibitors and substrates interfered with the beat frequency and with the percentage of motile spermatozoa. Whereas at low concentrations of BLT or aprotinin the beat frequency was more rapidly affected, while at high concentrations the percentage of motile spermatozoa decreased faster. In contrast to spermatozoa of sea urchin, carp, mammals which trypsin-like protease regulates the flagellar motility, studies on salmonid spermatozoa showed that a chymotrypsin-like protease regulates the sperm motility in an ATP concentration-dependent manner (Inaba and Morisawa, 1991; Inaba et al., 1993). Among different kinds of ATP-dependent proteases, Inaba et al., (1993) isolated and detected proteasomes (multicatalytic proteinase) with certain hydrolytic activity towards a substrate for chymotrypsin-like proteases along sperm flagella. In this study, complete inhibitory effects of both trypsin- and chymotrypsin-like inhibitors were not observed. This study showed that chymotrypsin-like inhibitor (TPCK) has stronger inhibitory effect than trypsin-like inhibitor (AGB) on motility of sturgeon spermatozoa at 1mM. Inaba and Morisawa (1992) showed that a chymotrypsin-like inhibitor (Suc-Leu-Leu-Val-Tyr-MCA) inhibited sperm motility at concentrations more than 0.1 mM. In case of se urchin and carp also very low concentrations more than 7.5 μM and more than 15 μM of aprotinin was needed for inhibiting sperm motility. Cosson and Gagnon (1988) found that beat frequency of carp and sea urchin spermatozoa is even high at 0.3 and 5 – 10 μM when sperm motility is less than 20 % in presence of aprotinin or BLT. The higher sperm velocity observed in presence of AGB and TPCK in this study can be related to mechanism regulating beating of flagella. Further studies are needed to understand real protease roles in activation of sturgeon spermatozoa by method of demebranation and reactivation of spermatozoa.

This study showed presence of both trypsin and chymotrypsin activities in sturgeon sperm, originated from sperm cells. Ciereszko et al., (1994) found that trypsin- and chymotrypsin- like inhibitors inhibit acrosin and/or acrosin-like activity in sturgeon sperm. However, the inhibitory effect is a species – specific characteristic and is a dose

dependent manner. In paddlefish and white sturgeon, AGB a trypsin-like inhibitor had stronger inhibitory effect on acrosin- like activity than TLCK or TPCK (chymotrypsin – like inhibitor) (Ciereszko et al., 2000). However, acrosin-like activity was more inhibited in white and lake sturgeon than paddlefish. High protease (mainly acrosin) activities found in spermatozoa cells compared to seminal plasma seem to be corresponded with presence of acrosome in the sperm cells. Previous studies showed that acrosin activity depend on several parameters such as concentration of spermatozoa, divalent cations (such as MgCl2, ZnCl2), temperature, incubation time of spermatozoa (Ciereszko et al., 1994, 1996). Effect of temperature on sturgeon acrosin-like activity is very similar to that on human acrosin at temperature over 20 oC. Acrosin activity in human (Kaminski et al., 1987) and sturgeon (Cierszko et al., 1996) spermatozoa can be inhibited by 50 % in presence of AGB (a trypsin-protease inhibitor) at 0.4 μM o.1 μM showing stronger inhibitory effect of AGB against acrosin activity of sturgeon spermatozoa than against.

In conclusion, both trypsin- (AGB) and chymotrypsin-like (TPCK) inhibitors inhibit sperm motility in dose dependent manner. Main trypsin-like activities of sturgeon sperm originates from spermatozoa cells, and very low protease activities detected from seminal plasma. Interestingly, this study showed that both trypsin and chymotrypsin- like inhibitor enhanced spermatozoa velocity at high concentrations (1 mM) compared to lower concentrations and controls. We also suppose that serine activity observed in sperm cells is acrosin or acrosin-like activity. Further studies are needed using molecular antibodies for confirmation and subsequently for investigation its role in fertilization mechanism.

Acknowledgements

This study was supported financially by USB RIFCH no. MSM6007665809, GACR no. 524/06/0817 and No. 303/06/0895 and in part by project 50520701 awarded by the Academy of Sciences of the Czech Republic.

References

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A., 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction 128, 819–828.

Alavi, S.M.H., Rodina, M., Cosson, J., Psenicka, M., Linhart, O., 2008. Roles of extracellular Ca^{2+} and pH on motility and flagellar wave form parameters in sturgeon spermatozoa. Cybium 32(2), 124–126.

Billard, R., Cosson, J., Fierville, F., Brun, R., Rouault, T., Williot, P., 1999. Motility analysis and energetics of the Siberian sturgeon *Acipenser baerii* spermatozoa. J. Appl. Ichthyol. 15 (4-5), 199–203.

Cechova, D., Töpfer-Petersen, E., Henschen, A., 1988. Boar proacrosin is a single-chain molecule which has the N-terminus of the acrosin A-chain (light chain). FEBS Lett. 241, 136–140.

Cechová, D., Jonáková, V., Veselský, L., Töpfer-Petersen, E., 1994. Serine protease activity in boar seminal vesicles and its immunological similarity to sperm acrosin. J. Reprod. Fertil. 100(2), 461–467.

Cherr, G.N., Clark, W.N.Jr., 1984. Acrosome reactions in sperm from the white sturgeon, *Acipenser transmontanus*. J. Exp. Zool. 232, 129–139.

Cherr, G.N., Clark, W.N.Jr., 1985. Gamete interaction in the white sturgeon *Acipenser transmontanus*: a morphological and physiological review. Env. Biol. Fish. 14, 11–22.

Ciereszko, A., 2008. Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity and cryopreservation success in fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G., (Eds.). Pp. 215–240. Alpha Science Ltd, Oxford.

Ciereszko, A., Dabrowski, K., Lin, F., 1994. Doroshov S. Identification of trypsin-like activity in sturgeon spermatozoa. J. Exp. Zool. 268, 486–491.

Ciereszko, A., Dabrowski, K., Mims, S.D., 2000. Glogowski J. Characteristics of sperm acrosin-like activity of paddlefish (*Polyodon spathula* Walbaum). Comp. Biochem. Physiol. B. 125, 197–203.

Ciereszko, A., Dabrowski, K., Ochkur, S.I., 1996. Characterization of acrosin-like activity of lake sturgeon (*Acipenser fulvescens*) spermatozoa. Mol. Reprod. Dev. 45(1), 72–77.

Cosson, M.P., Gagnon, C., 1988. Protease inhibitor and substrates block motility and microtubule sliding of sea urchin and carp spermatozoa. Cell Motil. Cytoskel. 10, 518–527.

Cosson, J., Linhart, O., 1996. Paddlefish, *Polyodon spathula,* spermatozoa: Effects of potassium and pH on motility. Folia Zool. 45, 361–370.

Cosson, J., Linhart, O., Mims, S., Shelton, W., Rodina, M., 2000. Analysis of motility parameters from paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) spermatozoa. J. Fish Biol. 56, 1348–1367.

Dabrowski, K., Ciereszko, A., 1994. Proteinase inhibitor(s) in seminal plasma of teleost fish. J Fish Biol. 45, 801–809.

de Lamirande, E., Gagnon, C., 1986. Effects of protease inhibitors and substrates on motility of mammalian spermatozoa. J. Cell Biol. 102, 1378–1383.

de Lamirande, E., Bardin, C.W., Gagnon, C., 1983. Aprotinin and a seminal plasma factor inhibit the motility of demembranated reactivated rabbit spermatozoa. Biol. Reprod. 28, 788–796.

Dettlaff, T.A., Ginsburg, A.S., Schmalhausen, O.I., 1993. Sturgeon Fishes: Developmental Biology and Aquaculture. Spring-Verlag, Berlin. Pp. 300

DiLauro, M.N., Kaboord, W., Walsh, R.A., 1998. Sperm-cell ultrastructure of North American sturgeons. I. The Atlantic sturgeon (*Acipenser oxyrhynchus*). Can. J. Zool. 76, 1822–1836.

Gagnon, C., Cosson, M.P., 1987. A ubiqitous protease regulates the motility of spermatozoa. Ann. NY Acad. Sci. 513, 589–591.

Gallis, J.L., Fedrigo, E., Jatteau, P., Bonpunt, E., Billard, R., 1991. Siberian sturgeon spermatozoa: Effects of dilution, pH, osmotic pressure, sodium and potassium ions on motility. In: *Acipenser*, Williot, P. (Ed.). Pp.143-51. Cemagref, Bordeaux,

Green, J.D., Summers, R.G., 1982. Effects of protease inhibitors on sperm-related events in sea urchin fertilization. Dev. Biol. 92, 139–144.

Hoshi, M., Numakunai, T., Sawada, H., 1981. Evidence for participation of sperm proteinase in fertilization of the solitary ascidians, *Halocynthia roretzi*: effects of protease inhibitors. Dev. Biol. 86, 117–121.

Inaba, K., Morisawa, M., 1991. A chymotrypsin-like protease is involved in motility of sperm in salmonid fish. Biomed. Res. 12, 435–437.

Inaba, K., Morisawa, M., 1992. Chymotrypsin-like protease activity associated with demembranated sperm of chum salmon. Biol. Cell 76, 329–333.

Inaba, K., Akazome, Y., Morisawa, M., 1993. Purification of proteasomes from salmonid fish serm and their localization along sperm flagella. J. Cell Sci. 104, 907–915.

Inaba, K., Morisawa, S., Morisawa, M., 1998. Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. J. Cell Sci. 111, 1105–1115.

Jamieson, B.G.M., 1991. Fish Evolution and Systematics: Evidence from Spermatozoa. Cambridge University Press, Cambridge. ISBN 0 521 41304 4.

Jelínková, P., Manásková, P., Tichá, M., Jonáková, V., 2003. Proteinase inhibitors in aggregated forms of boar seminal plasma proteins. Int. J. Biol. Macromol. 32 (35), 99–107.

Kaminski, J.M., Smith, D., Reid, D.S., Kennedy, W., Jeyendran, R.S., Zaneveld, L.J.D., 1987. Effect of aryl 4-guanidinobenzoates on the acrosin activity of human spermatozoa. Biol. Reprod. 36, 1170–1176.

Kowalski, R., Wojtczak, M., Glogowski, J., Ciereszko, A., 2003. Gelatinolytic and anti-trypsin activities in seminal plasma of common carp: relationship to blood, skin mucuc and spermatozoa. Aquat. Living Resour. 16: 438–444.

Kudo, S., 1991. Fertilization, cortical reaction, polyspermy-preventing and anti-microbial mechanisms in fish eggs. Bull. Inst. Zool. Acad. Sinica Monograph 16, 313–340.

Lahnsteiner, F., Patzner, R.A., 2008. Sperm morphology and ultrastructure in fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 1–61. Alpha Science Ltd, Oxford. 2008.

Lahnsteiner, F., Berger, B., Horvath, A., Urbanyi, B., 2004. Studies on the semen biology and sperm cryopreservation in the starlet, *Acipenser ruthenus* L. Aqua. Res. 35, 519–528.

Linhart, O., Mims, S.D., Shelton, W.L., 1995. Motility of spermatozoa from Shovelnose sturgeon, *Scaphirhynchus platorynchus*, and Paddlefish, *Polyodon spathula*. J. Fish Biol. 47, 902–909.

Polakoski, K.L., McRorie, R.A., Williams, W.L., 1973. Boar acrosin 1. purification and preliminary characterization of a proteinase from boar sperm acrosome. J. Biol. Chem. 248, 8178–8182.

Psenicka, M., Alavi, S.M.H., Rodina, M., Cicova, Z., Gela, D., Cosson, J., Nebesarova, J., Linhart, O., 2008. Morphology, chemical contents and physiology of chondrostean fish sperm: A comparative study between Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*). J. Appl. Ichthyol. 24, 371–377.

Psenicka, M., Alavi, S.M.H., Rodina, M., Gela, D., Nebesarova, J., Linhart, O., 2007. Morphology and ultrastructure of Siberian sturgeon, *Acipenser baerii*, spermatozoa using scanning and transmission electron microscopy. Biol. Cell 99 (2), 103–115.

Siegel, M.S., Polakoski. K.L., 1985. Evaluation of the human sperm proacrosin–acrosin system using gelatin–sodium dodecyl sulfate–polyacrylamide gel electrophesis. Biol. Reprod. 32, 713–720.

Toth, G.P., Ciereszko, A., Christ, S.A., Dabrowski, K., 1997. Objective analysis of sperm motility in the Lake sturgeon, *Acipenser fulvescens*: Activation and inhibition conditions. Aquaculture 154, 337–348.

Wojtczak, M., Glogowski, J., Koldras, M., Kucharczyk, D., Ciereszko, A., 2003. Charcterization of protease inhibitors of seminal plasma of cyprinids. Aquat. Living Resour. 16, 461–465.

Zaneveld, L.J.D., Chatterton, R.T., 1982. Biochemistry of mammalian reproduction. J. Wiley & Sons, New York. ISBN: 0471057312 . 580 P.

Zaneveld, L.J.D., Polakoski, K.L., Williams, W.L., 1973. A Proteinase and Proteinase Inhibitor of Mammalian Sperm Acrosomes. Biol. Reprod. 9, 219–225.

Chapter 6

Discussion

6.1. Sperm morphology and ultrastructure structure

Spermatozoon of species studied in present study is called “aquasperm” and composed of a head, a midpiece and a flagellum. To study morphology and ultrastructure of sperm, several techniques have been considered, mainly (a) electron microscopy, both scanning and transmission, (b) laser light-scattering spectroscopy and stroboscopic illumination (Van Look and Kime, 2003) and (c) computer-assisted morphometry analysis (ASMA) (Marco-Jimenez et al., 2008). Although, a regular or phase contrast microscopy can be used with a 100× objective (Tuset et al., 2008). Difficulties exist to compare the results of different studies on sperm morphology and ultrastructure, because (Lahnsteiner and Patzner, 2008; Psenicka et al., 2008); (a) each study may not describe an identical set of fine structural parameters. (b) Interpretation of the fine structural features are inconsistent depending on subjective criteria of the investigators. (c) Fixation and staining procedures may also play an important role in affecting comparability of results. And (d) the number of samples varies greatly between studies.

In Table 1, morphological and ultrastructural features of spermatozoa of species investigated in the present study are summarized and compared with some related species. In all species, spermatozoon is composed of a head, a midpiece and a flagellum. Spermatozoon of teleostean fishes is without acrosome, while spermatozoon of *Acipesner ruthenus* similar to other sturgeon fishes has an acrosomal complex at tip of the head (Psenicka et al., 2007, 2008). Head shape is elongated in sturgeons, but ovoid or roundish in teleosten fishes. This difference is may be due to fine structure of micropyle at the surface of eggs. Nucleus vesicles have been observed in *Barbus barbus* (Alavi et al., 2008b), *Tinca tinca* (Psenicka et al., 2006). Number of mitochondria highly differs among species, from 1 in *Perca fluviatilis* to 3-9 in Cyprinidae, and Acipenseridae. There is fin along the flagellum of *Acipenser* and *E. lucius*. In *Acipenser*, it was observed in both side of the flagellum, while in *Esox lucius*, it seems that it is only in one side. The fin structure seems to help sperm cells for moving during activation. It has been reported in almost all *Acipenser* species (DiLauro et al., 1999, 1998; Psenicka et al., 2007, 2008, 2009; Wei et al., 2007). *B. barbus* spermatozoon has the longest flagellum. Length of flagellum in Esocidae and *T. tinca* spermatozoa was significantly shorter than the other species. This study (Alavi et al., 2008c) showed that the morphological and ultrastructural characteristics of spermatozoa differ during the reproductive season. This is may be due to aging phenomenon in *B. barbus* spermatozoa towards the end of the reproductive season (Suquet et al., 1998).

Differences of morphology specially shape of head and the presence of fin along the flagellum seems to be depending on structure of eggs and reproductive biology of species. The structure of egg is important, because fertilization occurs when spermatozoon penetrates into the eggs through the “micropyle”. The present study again demonstrates that sperm morphology and ultrastructure can be used as a tool for systematics and phylogeny studies (Jamieson, 1991; Mattei, 1991; Lahnsteiner and Patzner, 2008). The morphological and ultrastructural differences are observed at family and species level. Nevertheless, inter-individual differences should be considered as observed in *T. tinca* (Psenicka et al., 2006), *B. barbus* (Alavi et al., 2008b), *E. lucius* (Alavi et al., 2009c) and *Acipenser* spp (Psenicka et al., 2007, 2008, 2009).

6.2. Seminal plasma characteristics

Seminal plasma has a unique composition containing substances supporting spermatozoa and plays a physio-endocrinological role after release of sperm from testes into the sperm duct and subsequenctly after release into the aquatic enivronemt (Ciereszko, 2008; Alavi et al., 2008a). Seminal plasma in most fishes is a secretory product of the testes and spermatic ducts (Lahnsteiner et al., 1993, 1994; Alavi et al., 2008a). Some components of seminal plasma originate from damaged spermatozoa and other somatic cells such as leukocytes, and cells of the testes and spermatic ducts (Ciereszko, 2008). Fish seminal plasma is characterized by a low protein concentration, containing mainly mineral compounds (sodium, potassium, calcium, magnesium) and low concentrations of other organic substances, such as hormones and pheromones, cholesterol, glycerol, vitamins, free amino acids, sugars, citric acid, and lipids. Information concerning the composition of seminal plasma and sperm physiology has been reviewed (Scott and Baynes, 1980; Linhart a al., 1991; Billard 1992, Billard et al., 1995a, 1995b; Cosson, 2004; Rurangwa et al., 2004; Ciereszko, 2008).

The main role of seminal plasma is to create an optimal environment for the storage of spermatozoa, very often for a prolonged time. Quantitaive charcteristics of sperm such as sperm viability (Flajshans et al., 2004), motility (Cosson 2008a,b; Rurangwa et al., 2004; Alavi and Cosson, 2005, 2006) and fertilizing ability (Billard et al., 1995a,b; Linhart et al., 2004, 2008) must be protected during storage time. In chondrostean fishes with acrosomal spermatozoa, the acrosome must also be preserved. Protection and sperm viability support mechanisms include: maintaining spermatozoa in the quiescent state (with the exception of fish with internal fertilization) through stable levels of osmolality, ionic concentrations, and/or sperm motility immobilizing proteins; supporting adequate levels of sperm nutrients for sperm metabolism, controlling and regulating final maturation processes, and protecting sperm against damage caused by proteolytic or oxidative attacks (Ciereszko, 2008). The role of seminal plasma probably extends beyond storage of sperm within the reproductive tract, it also may be important for external fertilization by creating a micro-environment for sperm movement by maintaining elevated osmolality and pH (Ciereszko, 2008).

pH, ionic composition (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-), osmolality, biochemical indices (such as total protein, cholestrol, total lipids), and enzymes (such as proteolytic enzymes) are main parameters that have been used for studying the qulity of the seminal plasma (Lahnsteiner et al., 1993, 1994; Billard et al., 1995a,b; Alavi et al., 2008a; Ciereszko, 2008). The results of the present study are summarized in Table 2 and compared with some related species. In all species, Na^+, Cl^- and K^+ are predominant ions in the seminal plasma. In chondreastean compared to teleostean, ions concentrations are lower, subsequently the osmolality show lower value (Table 2). The reason may be found in the specific secretory activities of the male reproductive organs with their especially designed sperm duct (Lahnsteiner et al., 1993, 1994, 1996; Ciereszko, 2008). In Cyprinidae (*Cyprinus Carpio*, *B. Barbus* and *Alburnus alburnus*) K^+ and Na^+ are higher and lower than the other teleosts. In general point of view, Na^+/ K^+ ratio in teleostean fishes is lower than in chondrostean fishes.

Table 1: Comparision of some morphological and ultrastructural charcteristics among some teleostean and chondrostean fishes.

Parameter	Cyprinidae					Esocidae		Percidae		Acipenseridae		
	Barbus barbus[1]			*Cyprinus carpio*[2]	*Tinca tinca*[3]	*Esox lucius*[4]	*E. niger*[5]	*Perca fluviatilis*[6]	*Sander lucioperca*[7]	*Acipenser ruthenus*[8]	*A. baerii*[9]	*A. sinensis*[10]
	March	April	May									
Length of acrosome	No	No	No	No	No	No	No	No	No	0.8	1.0	0.5
Width of acrosome										0.8	0.9	0.7
Head shape				roundish				ovoid			elongated	
Head length (µm)	1.6	1.7	1.6	3.3	1.3	1.3	1.8	1.9	1.6	3.3	5.0	4.5
Head width (µm)	1.7	1.8	1.7	2.5	1.7	1.4	1.9	1.8	1.3			
Midpiece length (µm)	0.5	0.5	0.4		1.0	1.0		0.9		1.0	1.1	2.2
Anterior width of Midpiece (µm)	0.9	0.9	0.8		0.8	0.7				0.7	0.9	0.6
Posterior width of Midpiece (µm)	0.5	0.5	0.5			0.5				0.9	1.1	1.8
Mitochondria Number		4-6		7-9	2-6			1		2-6	3-6	3-8
Flagellum length (µm)	46.5	54.3	51.7	43	25.4	30.1		30–35	30-35	42.5	44.8	33.3
Presence of fin		No		No	No	Yes		No	No	Yes	Yes	Yes
Nucleus vesicle (%)	1.6	0.8	0.8									
Total length (µm)	48.3	56.4	53.7		27.7	32.5	31.3			47.6	51.8	38.7

1- Alavi et al., (2008b), 2- Billard et al., (1995b), 3- Psenicka et al., (2006), 4- Alavi et al., (2009), 5- Linhart et al., (1991), Lahnsteiner et al., (1995), 7- Lahnsteiner and Mansour, (2004), 8- Psenicka et al., (2008, 2009), 9- Psenicka et al., (2007), 10- Wei et al., (2007).

Table 2: Ionic composition and osmolality of the seminal plasma in some models of teleostean and chondrostean fishes.

Species	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	Osmolality	pH	Authors
Barbus barbus	75.7 (1)	84.7 (1)	0.4 (1)		125.3 (1)	268 (1)		Alavi et al., (2008c)[1]
	69.7 (2)	84.0 (20	0.3 (2)		120.5 (2)	276 (2)		
	n.d.	n.d.	n.d.		n.d.	268 (3)		
Cyprinus carpio	71.3 (1)	78.9 (1)	10.7 (1)		110.6 (1)	346.0 (1)		Kruger et al., (1984)[2]
	59.0 (2)	73.0 (2)	11.5 (2)		96.3 (2)	290.3 (2)		
	58.1 (3)	77.6 (3)	8.5 (3)		102.1 (3)	297.8 (3)		
	75.0	82.4	2.0	0.8		302		Morisawa et al., (1983)
	51.3	43.5	0.7	0.27		286	8.0	Plouidy and Billard, (1982)
Tinca tinca	18.4	1.9	0.6	0.5		230		Linhart et al., (2003b)
Alburnus alburnus	66.6	40.6				266	8.5	Lahnsteiner et al., (1996)
Perca fluviatilis	124.0	10.2	0.7			283.9	8.3	Lahnsteiner et al., (1995)
	131.0	10.7	2.4		106.8	298.1		Alavi et al., (2007)
Esox lucius	115.5	24.8	1.1		116.2	272.6-283.9		Hulak et al., (2008a,b)
								Alavi et al., (2009c)
Acipenser ruthenus	20.1	1.3	0.2		6.1	50.7	8.1	Psenicka et al., (2008)
Acipenser baerii	28	2.5				38		Gallis et al., (1991)
	31.4	3.5	0.2		14.0	77.2	8.2	Psenicka et al., (2008)
Acipenser persicus	62.4 (1)	6.9 (1)	0.8 (1)		21.1 (1)	82.6 (1)		Alavi et al., (2004, 2006)[3]
	50.3 (2)	5.8 (2)	0.8 (2)		9.0 (2)	55.1 (2)		
	52.5 (3)	7.5 (3)	0.8 (3)		12.3 (3)	56.5 (3)		
Polyodon spathula	4.0 (1)	0.4 (1)	0.9 (1)					Linhart et al., (2003a)[4]
	4.7 (2)	1.1 (2)	0.7 (2)					
	6.0 (3)	1.3 (3)	0.5 (3)					

[1]Data of 1, 2 and 3 represents, March, April and May.
[2]Data of 1, 2 and 3 represents, winter, early spring and Late spring. Ca^{2+} is measured in mg 100 ml^{-1}
[3]Data of 1, 2 and 3 represents first, second and third stripping with 12 h intervals.
[4]Data of 1, 2 and 3 represent first, second and third stripping at 1, 2 and 3 day post injection, respectively.

Osmolality of teleostean freshwater fish is approximately 300 (230–346) mOsmol kg^{-1}). In sturgeon (*Acipenser* spp. and *Polyodon spathula*), the osmolality of seminal plasma is in range of 38–82.6 mOsmol kg^{-1}. The ionic factors and osmolality of the seminal plasma did not change in *B. Barbus* during the reproductive season, but significantly changed in *C. Carpio* (Kruger et al., 1984). Time from spermiation induction to collect the sperm also influence the seminal plasma compositions and osmolality as has been reported in *A. Persicus* (Alavi et al., 2006) and *P. spathula* (Linhart et al., 2003a). Very low concentrations of ions in *T. Tinca* (Linhart et al., 2003) can be due to contamination of sperm by urine which affect the composition and osmolality of the seminal plasma (Perchec-Poupard, et al., 1998; Dreanno et al., 1998; Suquet et al., 1998). Feeding regim also affect the secretory activities of the male reproductive organs (Alavi et al., 2009a).

6.3. Sperm production indices: Sperm volume and concentration

Sperm volume in chondrostean is usually higher than teleostean fishes, but spermatozoa concentration in lower. This study showed that sperm volume decreased in *B. Barbus* (Alavi et al., 2008c) towards the end of the reproductive season, but increased in *P. fluviatilis* (Alavi et al., 2009d) similar to that of *Salmo salar m. Sebago* (Piironen, 1985). Changes of sperm volume during the reproductive season can be related to steroid hormones (such as testosterone or 11-ketotestosterone) which regulate the final maturation of sperm in sperm duct (Nagahama, 1994; Billard et al., 1995a,b; Miura and Miura, 2003)

Spermatozoa concentration is also an important factors for determination of sperm quality (Suquet et al., 1994; Billard et al., 1995a). Spermatozoa concentartion can be correlated with body size othe the males. In this study, significant positive correlations between spermatozoa concentration – length and – weight of *B. Barbus* males. But, significant negative trends with increasing fish size were reported in brown trout (Poole and Dillane, 1998) and Atlantic salmon (Daye and Glebe, 1984; Vladic and Jarvi, 2001). By increasing of broodstock age, the concentration of sperm decreased in sockeye salmon (Hoysak and Liley, 2001), Atlantic salmon (Vladic and Jarvi, 2001) and brown trout (Poole and Dillane, 1998). The environmental conditions during broodstock cultivation or sperm collection from migratory fish species are important in sperm release process from testis and subsequently affect spermatozoa concentration. Krol et al., (2006) observed lower concentration of sperm in Eurasian perch those kept at high water temperature at the beginning of spawning period. Spermation induction by injection of sexual hormones such as carp pituitary extract, ovopel, HCG, GnRHa and LHRHa influence on spermatozoa concentration (Billard et al., 1995a; Yaron, 1995). All of methods used in inducing of spermiation caused to hydration of the testis during sperm release (Nagahama, 1994; Miura and Miura, 2003) and subsequently affect spermatozoa concentration as well seminal plasma and sperm motility parameters (Alavi and Cosson, 2005, 2006). During the reproductive season, spermatozoa concentration may change due to changes of hormonal regulation of spermiation (Billard, 1992; Billard et al., 1995a,b). Spermatozoa concentration has been shown to increase throughout the spawning season for Atlantic halibut (Methven and Crim, 1991), turbot (Suquet et al., 1998) and Atlantic salmon (Piironen, 1985), but decrease throughout the spawning season in rainbow trout

(Buyukhatipoglu and Holtz, 1984; Munkittrick and Moccia, 1987; Billard, 1992), sea bass (Kara and Labed, 1994), common barbel (Alavi et al., 2008c) and perch (Alavi et al., 2009d).

Table 2: Spermatozoa concentration in some models of teleostean and chondrostean fishes.

Species	Spermatozoa concentration $\times 10^9$ spermatozoa ml^{-1}	Authors
Barbus barbus	18.8 (1)	Alavi et al., (2008c)
	11.8 (2)	
	12.5 (3)	
Cyprinus carpio	9.6–27.4	Christ et al., (1996)
Tinca tinca	0.6–3.3	Caille et al., (2006)
	2.1	Linhart et al., (2003b)
	76.2	Piironen and Hyvarinen (1983)
Perca fluviatilis	59.2 (1)	Alavi et al., (2009)[1]
	66.5 (2)	
	36.0 (3)	
	45.45 (4)	
	29.2	Alavi et al., (2007)
	17.0	Lahnsteiner et al., (1995)
Esox lucius	22.6	Hulak et al., (2008a,b)
Acipenser ruthenus	0.4	Psenicka et al., (2008)
	0.5–0.6	Piros et al., (2002)
Acipenser baerii	0.6	Psenicka et al., (2008)
	1.7 – 2.4	Piros et al., 2002
	8.3 (1)	Alavi et al., (2006)[2]
	6.5 (2)	
	3.5 (3)	

[1]Data of 1, 2 and 3 represents, March, April and May.
[2]Data of 1, 2 and 3 represents first, second and third stripping with 12 h intervals.

Very little detailed information is available about the correlation between sperm density and motility. No effect of spermatozoa concentrtation on sperm motility has been reported by Tvedt et al., (2001), Alavi et al., (2005) and Alavi et al., (2009b) in Atlantic halibut, Persian sturgeon and common barbel. Rideout et al., (2004) observed significant negative relationship between sperm motility and spermatocrit only in samples with spermatocrits >70%. Positive correlation between sperm density or spermatocrit and fertilizing ability has been reported in Atlantic herring (Rosenthal et al., 1988), common carp (Koldras and Mejza, 1983), and brown trout (Poole and Dillane, 1998).

6.4. Sperm motility

6.4.1. Dynamics of sperm motility parameters during activation

Sperm of studied species similar to other freshwater fishes (Cosson et al., 1999; Morisawa et al., 1999; Cosson, 2004; Alavi and Cosson, 2006) was immotile in the seminal plasma and its motility was triggered in a hypo-osmotic condition. Sperm motility in the seminal plasma was inhibited due to high osmolality in teleostean fishes

similar to most teleostean freshwater fish (Morisawa et al., 1983; Billard et al., 1995a; Morisawa, 2008). High concentration of K^+ prohibited sperm motility in the seminal plasma of *A. ruthenus* similar to salmonids (Billard, 1992; Billard and Cosson, 1992) and other sturgeon species (Gallis et al., 1991; Billard et al., 1999; Alavi et al., 2004). Duration of sperm motility was very short in teleostean fishes (less than 1 min in distlled water) compared to *A. ruthenus* (longer than 2 min in distilled water). All parameters of sperm motility (spermatozoa velocity, percentage of motility and beat frequency) decreased rapidly after triggering of sperm motility. These decreases are due to decrease of ATP content of spermatozoa, which occur rapidly after activation of sperm motility (Perchec et al., 1995; Billard et al., 1999; Cosson, 2004). However, the velocity of the spermatozoa vary among species. The spermatozoa velocity after activation in distlled water were measured 95 – 105 in *B. barbus* (Alavi et al., 2009b), 105 – 125 in *P. fluviatils* (Alavi et al., 2007), 175 – 185 in *E. lucius* (Alavi et al., 2009c) and 160 – 180 in *A. ruthenus* (Alavi et al., 2008). Spermatozoa velocity depends on several parameters such as head dimension (diameter of head), beat frequency, length of flagellum, physical parameters of wave propagation such as wave length and amplitude Cosson et al., 2000; Alavi et al., 2008d). Higher spermatozoa velocity in E. lucius and A. ruthenus can be due to presence of fin along the flagellum, plays an important role for wave efficiency and subsequently sperm velocity. The fin(s) structure along the flagellum have been also observed in marine fishes (Cosson et al., 2008a,b) and other sturgeons (DiLauro et al., 1998, 1999, Psenicka et al., 2007, 2008, 2009).

6.4.2. Factors affecting sperm motility

It has been already shown in the literature that there are several correlations between seminal plasma composition and sperm motility in some species; Atlantic salmon, *Salmo salar* (Hwang and Idler, 1969), common carp, *Cyprinus carpio* (Kruger et al., 1984), bleak, *Alburnus alburnus* (Lahnsteiner et al., 1996), rainbow trout, *Oncorhynchus mykiss* (Lahnsteiner et al., 1998), Persian sturgeon, *Acipenser persicus* (Alavi et al., 2004) and chinook salmon, *Oncorhynchus tshawytscha* (Rosengrave et al., 2009). Positive correlations between Na^+ and osmolality of seminal plasma with percentage of sperm motility have been reported in some freshwater teleosts such as *B. barbus* (Alavi et al., 2009b). In contrast, negative correlations between K^+ and sperm motility have been reported (Billard and Cosson, 1992; Lahnsteiner et al., 1996; Alavi et al., 2009b). Such relationships between ionic composition and sperm motility were not found in some species such as Chinook salmon, *O. tshawytscha* (Rosengrave et al., 2009) and Persian sturgeon, *Acipenser persicus* (Alavi et al., 2004).

It has been shown that external factors (predilution of spermatozoa in immobilizing medium, ions (K^+, Na^+, and Ca^{2+}) and osmolality of the seminal plasma) affect sperm motility parameters. These effects depend on several parameters such as spermatozoa concentration, ionic composition and osmolality of the seminal plasma, contamination of sperm by urine, aging of sperm (Billard et al., 1995a,b; Suquet et al., 1994, 1998; Cosson et al., 1999; Alavi and Cosson, 2006). The optimum sperm motility parameters were observed in osmolalities (mOsmol kg^{-1}) between 100–200 for *P. fluviatlis* (Alavi et al., 2007), 125–235 for *E. lucius* (Alavi et al., 2009c), 200–235 for *B. Barbus* (Alavi et al., 2009b) and 25–50 for *A. ruthenus* (Alavi et al., unpublished data). In

teleostean species, osmolality higher than 200–250 mOsmol kg^{-1} significantly decreased both percentage of motility and spermatozoa velocity. In *A. ruthenus*, it was observed at osmolality 75–100 mOsmol kg^{-1} (Alavi et al., unpublished data). However, the osmolality, which totally supresses the sperm motiliy vary among species. In *B. barbus* (Alavi et al., 2009b), *P. fluviatilis* (Alavi et al., 2007), *E. lucius* (Alavi et al., 2009c), *A. ruthenus* (Alavi et al., unpublished data), the motility of sperm was inhibited at 330–350, 300, 375, and 150–175 mOsmol kg^{-1}. Our studies also showed that lower velocity of spermatozoa after activation in distilled water can be related to hypo-osmotic shock on flagella during sperm activation. In all teleostean species, blebs appeared along the flagellum at 30 s post sperm activation, which prevent correct and efficient wave propagation. The tip of the flagellum become curled into a loop shape which shorten the flagellum (Cosson et al., 2000; Cosson et al., 2008a,b; Linhart et al., 2008; Alavi et al., 2009b,c). Linhart et al., (2008) illustrated that incubation of spermatozoa after activation in distilled water or hypo-osmotic condition in a K^+-rich incubation medium can mitigate the structural damages and subsequently may help spermatozoa to increase their motility and fertilization.

6.4.2. Flagellar waveform and parameters

When sperm is observed a few second after activation, the waves propagte the whole length of the flagellum. A few second after activation, these waves are restrictaed to the part of the flagellum close to the head. Close to the end of sperm motility, only a very restricated portion of the flagellum in the vicinity of the head will exhibit wave. The ending process is the full absence of wave leading to immotility. Size and shape of the waveform (flagellar beating) is determined by the inner arms activities to generate flagellar bends in the axoneme; the outer dynein arms add power and increase beat frequency (Brokaw and Kamiya, 1987) and inner arm dynein plays a distinct role in generation and control of motility (Porter and Sale, 2000). The central pair of microtubules and radial spokes interact with the inner arms to control the flagellar waveform (Smith and Lefebvre, 1997). Studies on *E. lucius* sperm (Alavi et al., 2009) showed that the number of waves and curvatures differed significantly in relation to osmolality of the activation media. At the same osmolality, wave length and amplitude were decreased, but number of curvatures and waves increased as a function of time post activation. It was also demonstrated that ATP induces sliding between specific subsets of doublet microtubules and that Ca^{2+} ions induce changes in waveform (Wargo et al., 2004). Our study on *A. ruthenus* sperm (Alavi et al., 2008d) showed that Ca2+ concentration in the activation medium influence and subsequently change the flagellar beating parameters.

In these studies, flagellar beating was symmetric, but Ca^{2+} at high alkalinity induced assymetric behavior of sperm in sturgeons (Alavi et al., 2009d). Ca^{2+} has been shown to play an important role in modulating the waveforms of the flagella by the specific activation of the dynein arms (Brokaw, 1979; Mizuno et al., 2009). Changes in the direction of sperm movement are caused by modulation of the beating of the flagellar wave; a process that involves Ca^{2+}- dependent changes in flagellar assymetry (Brokaw, 1979). Certain Ca^{2+}- binding proteins (such as calaxin, Mizuno et al., 2009) may be components or associated proteins of outer arm dynein (Inaba, 2008), which may be also

involved in the asymmetry of axonemal movement.

6.4.3. Protease activities in sturgeon sperm: their effects on sperm motility

Sturgeon spermatozoa exhibit an acrosin- like activity (Ciereszko et al., 1996, 2000), which is in contrast to teleost sperm with no acrosin activity and even anti-proteinase (including anti-acrosin) activity (Dabrowski and Ciereszko, 1994; Kowalski et al., 2003; Wojtczak et al., 2003). In our study (article 9), the protease activity was significantly higher in sperm extract than seminal plasma (Alavi et al., 2009). In samples of sperm acidic extract, total protease and serine activities were accumulated in protein band with relative molecular mass of 33kDa, which seems to be an acrosin. Effects of protease inhibitors on protease activity of sturgeon sperm seems to be a species–specific characteristic and a dose dependent manner. In paddlefish and white sturgeon, AGB a trypsin-like inhibitor had stronger inhibitory effect on acrosin- like activity than TLCK or TPCK (chymotrypsin – like inhibitor) (Ciereszko et al., 2000). However, acrosin-like activity was more inhibited in white and lake sturgeon than paddlefish. However, previous studies showed that acrosin activity depend on several parameters such as concentration of spermatozoa, divalent cations (such as MgCl2, ZnCl2), temperature and incubation time of spermatozoa (Ciereszko et al., 1994, 1996). Further studies are needed to purify and isolate the protease from sturgeon sperm cells for better unedrstanding of acrosomal reaction of sperm and its role during fertilziation.

Previous studies (Gagon and Cosson, 1987; Cosson and Gagnon, 1988) showed that protease inhibitors and substrates interfered with the beat frequency and with the percentage of motile spermatozoa. Studies on A. ruthenus sperm reveals that high concentrations of protease inhibitors required for giving complete inhibition of sturgeon sperm motility similar to that of sea urchin and carp (Gagon and Cosson, 1987; Cosson and Gagnon, 1988). In our study on *A. ruthenus* sperm (Alavi et al., 2009) showed that both trypsin- (AGB) and chymotrypsin- like (TPCK) inhibitors had significant effect on sperm motility in dose and time dependent manner. Percentage of motile spermatozoa significantly decreased when AGB or TPCK was added to activation medium >0.25 mM or >0.5 mM. TPCK inhibitory effect was more effective than AGB at 120 sec post activation. Sperm velocity was significantly higher when AGB and TPCK concentrations were >0.25 and 1 mM. In contrast, studies on salmonid spermatozoa showed that a chymotrypsin-like protease regulates the sperm motility in an ATP concentration-dependent manner (Inaba and Morisawa, 1991, 1992; Inaba et al., 1993). Taken together, more studies are necessary for finding if proteasomes are involved in sperm motility of sturgeon by demebranation and reactivation of spermatzoa in presence of protease inhibitors.

References

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes: (I) Effects of pH and temperature. Cell Biol. Int. 29, 101–110.

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes: (II) Effects of ions and osmolality. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A., 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction 128, 819–828.

Alavi, S.M.H., Cosson, J., Kazemi, R., 2006. Semen characteristics in *Acipenser persicus* in relation to sequential stripping. J. Appl. Ichthyol. 22 (Suppl. 1), 400–405.

Alavi, S.M.H., Linhart, O., Coward, K., Rodina, M., 2008a. Fish Spermatology: Implication for aquaculture management. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 397–460. Alpha Science Ltd, Oxford, UK.

Alavi, S.M.H., Pšenička, M., Policar, T., Rodina, M., Hamáčková, J., Kozák, P., Linhart, O., 2009a. Sperm quality in male *Barbus barbus* L. fed different diets during the spawning season. Fish Physiol. Biochem. In press.

Alavi, S.M.H., Psenicka, M., Policar, T., Linhart, O., 2008b. Morphology and fine structure of *Barbus barbus* (Teleostei: Cyprinidae) spermatozoa. J. Appl. Ichthyol. 24, 378–381.

Alavi, S.M.H., Psenicka, M., Rodina, M., Policar, T., Linhart, O., 2008c. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Cyprinidae: Teleostei) during the reproductive season. Aquat. Living Resour. 21, 75 – 80.

Alavi, S.M.H., Rodina, M., Cosson, J., Psenicka, M., Linhart, O., 2008d. Roles of extracellular Ca^{2+} and pH on motility and flagellar wave form parameters in sturgeon spermatozoa. Cybium 32(2), 124–126.

Alavi, S.M.H., Rodina, M., Policar, T., Linhart, O., 2009b. Relationships between semen characteristics and body size in *Barbus barbus* L. (Teleostei: Cyprinidae) and effects of ions and osmolality on sperm motility. Comp. Biochem. Physiol A. In press.

Alavi, S.M.H., Rodina, M., Policar, T., Kozak, P., Psenicka, M., Linhart, O. 2007. Semen of *Perca fluviatilis* L: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology, 68, 276–283.

Alavi, S.M.H., Rodina, M., Viveiros, A.T.M., Cosson, J., Gela, D., Boryshpolets, S., Linhart, O., 2009c. Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius* L.). Theriogenology. In press.

Alavi, S.M.H., Rodina, M., Hatef, A., Stejskal, V., Policar, T., Hamáčková, J., Linhart, O., 2009d. Sperm motility and monthly variations of semen characteristics in *Perca fluviatilis* (Teleostei: Percidae). Czech J. Animal Sci. Submitted.

Billard, R., 1992. Reproduction in rainbow trout: sex differentiation, dynamics of gametogenesis, biology, and preservation of gametes. Aquaculture, 100, 263–298.

Billard, R., Cosson, M.P., 1992. Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool., 261, 122–131.

Billard, R., Cosson, J., Crim, L.W., Suquet, M., 1995a. Sperm physiology and quality. In: *Brood stock management and egg and larval quality,* Bromage, N.R., Roberts, R.J., (Eds.). Pp. 25–52. Blackwell Science, Amsterdam.

Billard, R., Cosson, J., Fierville, F., Brun, R., Rouault, T., Williot, P., 1999. Motility analysis and energetics of the Siberian sturgeon *Acipenser baerii* spermatozoa. J. Appl. Ichthyol. 15 (4-5), 199–203.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995b. Biology of sperm and artificial reproduction in carp. Aquaculture 124, 95–112.

Brokaw, C.J., 1979. Calcium-induced asymmetrical beating of triton-demembranated sea urchin sperm flagella. J. Cell Biol. 82, 401–411.

Brokaw, C.J., Kamiya, R., 1987. Beding patterns of Chlamydomonas flagella. IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. Cell Motil. Cytoskel. 8, 68–75.

Buyukhatipoglu, S., Holtz, W., 1984. Sperm output in rainbow trout (*Salmo gairdneri*). Effect of age, timing, and frequency of stripping and presence of females. Aquaculture 37, 63–71.

Caille, N., Rodina, M., Kocour, M., Gela, D., Flajshans, M., Linhart, O., 2006. Quantity, motility and fertility of tench *Tinca tinca* (L.) sperm in relation to LHRH analogue and carp pituitary treatments. Aqua. Int. 14, 75–87.

Christ, S.A., Toth, G.P., McCarthy, H.W., Torsella, J.A., Smith, K.M., 1996. Monthly variations in sperm motility in common carp assessed using computer-assisted sperm analysis (CASA). J. Fish Biol. 48, 1210–1222.

Ciereszko, A., 2008. Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity and cryopreservation success in fish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, R., Rafiee, G. (Eds.). Pp. 215–240. Alpha Science Ltd, Oxford.

Ciereszko, A., Dabrowski, K., Lin, F., 1994. Doroshov S. Identification of trypsin-like activity in sturgeon spermatozoa. J. Exp. Zool. 268, 486–491.

Ciereszko, A., Dabrowski, K., Mims, S.D., 2000. Glogowski J. Characteristics of sperm acrosin-like activity of paddlefish (*Polyodon spathula* Walbaum). Comp. Biochem. Physiol. B. 125, 197–203.

Ciereszko, A., Dabrowski, K., Ochkur, S.I., 1996. Characterization of acrosin-like activity of lake sturgeon (*Acipenser fulvescens*) spermatozoa. Mol. Reprod. Dev. 45(1), 72–77.

Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquac Int. 12, 69–85.

Cosson, J., 2008a. Methods to analyse the movements of fish spermatozoa and their flagella. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 63–102. Alpha Science Ltd, Oxford, UK.

Cosson, J., 2008b. The motility apparatus of fish spermatozoa. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.). Pp. 281–316. Alpha Science Ltd, Oxford, UK.

Cosson, M.P., Gagnon, C., 1988. Protease inhibitor and substrates block motility and microtubule sliding of sea urchin and carp spermatozoa. Cell Motil. Cytoskel. 10, 518–527.

Cosson, J., Billard, R., Cibert, C., Dreanno, C., Suquet, M., 1999. Ionic factors regulating the motility of fish sperm. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Ed.). Pp. 161–186. Cache Rive Press. Vienna IL.

Cosson, J., Groison, A-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008a. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460–486.

Cosson, J., Groison, A-L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008b. Marine fish spermatozoa: racing ephemeral swimmers. Reproduction 136, 277–294.

Cosson, J., Linhart, O., Mims, S., Shelton, W., Rodina, M., 2000. Analysis of motility parameters from paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) spermatozoa. J. Fish Biol. 56, 1348–1367.

Daye, P.G., Glebe, B.D., 1984. Fertilization success and sperm motility of Atlantic salmon (*Salmo salar* L.) in acidified water. Aquaculture, 43, 307–312.

DiLauro, M.N., Kaboord, W., Walsh, R.A., 1998. Sperm-cell ultrastructure of North American sturgeons. I. The Atlantic sturgeon (*Acipenser oxyrhynchus*). Can. J. Zool. 76, 1822–1836.

DiLauro, M.N., Kaboord, W.S., Walsh, R.A., 1999. Sperm-cell ultrastructure of North American sturgeons. II. The shortnose sturgeon (*Acipenser brevirostrum*, Lesueur, 1818). Can. J. Zool.-Rev. Can. Zool. 77, 321–330.

Dreanno, C., Suquet, M., Desbruyeres, E., Cosson, J., Le Delliou, H., Billard, R., 1998. Effect of urine on semen quality in Turbot, *Psetta maxima*; Aquaculture, 169, 247–262.

Flajšhans, M., Cosson, J., Rodina, M., Linhart, O., 2004. The application of image cytometry for viability assessment of dual fluorescent - stained spermatozoa of fish. Cell Biol. Int. 28, 12: 955–959.

Gagnon, C., Cosson, M.P., 1987. A ubiqitous protease regulates the motility of spermatozoa. Ann. NY Acad. Sci. 513, 589–591.

Gallis, J.L., Fedrigo, E., Jatteau, P., Bonpunt, E., Billard, R., 1991. Siberian sturgeon spermatozoa: Effects of dilution, pH, osmotic pressure, sodium and potassium ions on motility. In: *Acipenser*, Williot, P. (Ed.). Pp.143-51. Cemagref, Bordeaux,

Hoysak, D.J., Liley, N.R., 2001. Fertilization dynamics in sockeye salmon and a comparison of spem from alternative male phenotypes. J. Fish Biol. 58, 1286 – 1300.

Hulak, M., Rodina, M., Alavi, S.M.H., Linhart, O., 2008a. Evaluation of semen and urine of pike (*Esox lucius* L.): Ionic compositions and osmolality of the seminal plasma and sperm volume, density and motility. Cybium 32(2), 189–190.

Hulak, M., Rodina, M., Linhart, O., 2008b. Characteristics of stripped and testicular Northern pike (*Esox lucius*) sperm: spermatozoa motility and velocity. Aquat. Living Resour. 21, 207–212.

Hwang, P.C., Idler, D.R., 1969. A study of major cations, osmotic pressure, and pH in seminal components of Atlantic salmon. J. Fish. Res. Board Can. 26, 413–419.

Inaba, K., 2008. Molecular mechanisms of the activation of flagellar motility in sperm. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, R., Rafiee, G., (Eds.). Pp. 267–279. Alpha Science Ltd, Oxford.

Inaba, K., Morisawa, M., 1991. A chymotrypsin-like protease is involved in motility of sperm in salmonid fish. Biomed. Res. 12, 435–437.

Inaba, K., Morisawa, M., 1992. Chymotrypsin-like protease activity associated with demembranated sperm of chum salmon. Biol. Cell 76, 329–333.

Inaba, K., Akazome, Y., Morisawa, M., 1993. Purification of proteasomes from salmonid fish serm and their localization along sperm flagella. J. Cell Sci. 104, 907–915.

Jamieson, B.G.M., 1991. *Fish Evolution and Systematics: Evidence from Spermatozoa*. Cambridge University Press, Cambridge. ISBN 0 521 41304 4.

Kara, M.H., Labed, S., 1994. Evolution des caracte´ristiques du sperme de *Sparus aurata* et *Dicentrarchus labrax* au cours d'une saison de reproduction. *Cahiers de Biologie Marine* 35, 281–288.

Koldras, M., Mejza, T., 1983. Effects of quantity and quality of carp sperm on egg fertilization success. Acta Ichthyol. Piscatoria, 13, 83–91.

Kowalski, R., Wojtczak, M., Glogowski, J., Ciereszko, A., 2003. Gelatinolytic and anti-trypsin activities in seminal plasma of common carp: relationship to blood, skin mucuc and spermatozoa. Aquat. Living Resour. 16: 438–444.

Krol, J., Glogowski, J., Demska-Zakes, K., Hliwa, P., 2006. Quality of semen and histological analysis of testis in *Perca fluviatilis* L. during spawning period. Czech J. Animal Sci. 51, 220–226.

Kruger, J.C., Smith, G.L., Van Vuren, J.H.J., Ferreira, J.T., 1984. Some chemical and physical characterestics of the semen of *Cyprinus carpio* and *Oreochromis mossambicus*. J. Fish Biol., 24, 263–272.

Lahnsteiner, F., Mansour, N., 2004. Sperm fine structure of the pikeperch, *Sander lucioperca* (Percidae, Teleostei). J. Submicrosc. Cytol. Pathol. 36,309–312.

Lahnsteiner, F., Patzner, R.A., 2008. Sperm morphology and ultrastructure in fish. In: *Fish Spermatology,* Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G., (Eds.). pp. 1–61. Alpha Science Ltd, Oxford.

Lahnsteiner, F., Patzner, R.A., Weismann, T., 1993. The spermatic ducts of salmonid fishes (Salmonidae, Teleostei). Morphology, histochemistry and composition of the secretion. J. Fish Biol. 42, 79–93.

Lahnsteiner, F., Patzner, R.A., Weismann, T., 1994. The testicular main ducts an spermatic ducts in some cyprinid fishes. II. Composition of the seminal fluid. J. Fish Biol. 44, 459–467.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1995. Fine structure and motility of spermatozoa and composition of seminal plasma in the perch. J. Fish Biol. 47, 492 – 508.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1996. Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. Fish Physiol. Biochem. 15, 167–179.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture 163, 163–181.

Lahnsteiner, F., Mansour, N., Berger, B., 2004. Seminal plasma proteins prolong the viability of rainbow trout (*Oncorynchus mykiss*) spermatozoa. Theriogenology 62, 801–808.

Linhart, O., Alavi, S.M.H., Rodina, M., Gela, D., Cosson, J., 2008. Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (*Cyprinus carpio*). J. Appl. Ichthyol. 24, 386–392.

Linhart, O., Mims, S.D.,Gomelsky, B., Hiott, A.E., Shelton, W.L., Cosson, J., Rodina, M., Gela, D., Bastl, J., 2003a. Ionic and osmolality of paddlefish (*Polyodon spathula,* Acipenseriformes) seminal fluid with motility parameters. Aqua. Int. 11, 357–368.

Linhart, O., Rodina, M., Gela, D., Kocour, M., 2004. Optimalization of artificial propagation in European catfish, *Silurus glanis* L. Aquaculture 235, 619–632.

Linhart, O., Rodina, M., Bastl, J., Cosson J., 2003b. Urinary bladder, ionic composition of seminal fluid and urine with characterization of sperm motility in tench (*Tinca tinca* L.). Journal of Appl. Ichtyol.,19, 177–181.

Linhart, O., Slechta, V., Slavik, T., 1991. Fish sperm composition and biochemistry. Bull. Inst. Zool. Acad. Sinica Monograph 16, 285–311.

Marco-Jimenez, F., Penaranda, D.S., Perez, L., Viudes-de-Castro, M.P., Mylonas, C.C., Jover, M., Asturiano, J.F., 2008. Morphometric characterization of sharpsnout sea bream (*Diplodus puntazzo*) and gilthead sea bream (*Sparus aurata*) spermatozoa using computer-assisted spermatozoa analysis (ASMA). J. Appl. Ichthyol. 24, 382–385.

Mattei, X., 1991. Spermatozoon ultrastructure and its systematic implications in fishes. Can. J. Zool. 69, 3038–3055.

Methven, D.A., Crim, L.W., 1991. Seasonal changes in spermatocrit, plasma sex steroids, and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*). In *Proceedings of the Fourth International Symposium on Reproductive Physiology of Fish* (Scott, A. P., Sumpter, J. P., Kime, D. E. & Rolfe, M. S., eds), p. 170. Sheffield: FishSymp 91.

Miura, T., Miura, C., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem. 28, 181–186.

Morisawa, M., 2008. Adaptation and strategy for fertilization in the sperm of teleost fish. J. Appl. Ichthyol. 24, 362–370.

Morisawa, M., Oda, S., Yoshida, M., Takai, H., 1999. Transmembrane signal transduction for the regulation of sperm motility in fishes and ascidians. In: *The male gamete: From basic to clinical applications*, Gagnon, C., (Eds.). pp. 149–160. Cache Rive Press. Vienna, II.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., Yasuda, K., 1983. Effect of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. J. Exp. Biol. 107, 95–103.

Munikittrick, K.R., Moccia, R.D., 1987. Seasonal changes in the quality of rainbow trout 9Salmo gairdneri) semen: effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma constitutions. Aquaculture 64, 147–156.

Nagahama, Y., 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217–229.

Perchec, G., Jeulin, C., Cosson, J., Andre, F., Billard, R., 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747–753.

Perchec-Poupard, G., Paxion, C., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1998. Initiation of carp spermatozoa motility and early ATP reduction after milt contamination by urine. Aquaculture 160: 317–328.

Piironen, J., 1985. Variation in the properties of milt from the Finnish landlocked salmon (*Salmo salar m. Sebago* Girard) during a spawning season. Aquaculture, 48, 337–350.

Piros, B., Glogowski, J., Kolman, R., Rzemieniecki, A., Domagala, J., Horvath, A., Urbanyi, B., Ciereszko, A., 2002. Biochemical characterization of Siberian sturgeon *Acipenser baeri* and starlet, *Acipenser ruthenus*, milt plasma and spermatozoa. Fish Physiol. Biochem. 26, 289–295.

Plouidy, M.G., Billard, R., 1982. The chemical composition fluids of the gametes in the common carp, *Cyprinus carpio*,. In: *Reproductive Biology of Fishes*, Richter, C.J.J., Goos, H.J.Th. (Eds.). p. 134. PUDOC, Wagenington.

Poole, W.R., Dillane, M.G., 1998. Estimation of sperm concentration of wild and reconditioned brown trout, Salmo trutta L. Aqua. Res. 29, 439–445.

Psenicka, M., Vancova, M., Koubek, P., Tesitel, J. and Linhart, O., 2009. Fine structure and morphology of sterlet (*Acipenser ruthenus* L. 1758) spermatozoa and acrosin localization. Anim. Reprod. Sci. 111: 3–16.

Psenicka, M., Alavi, S.M.H., Rodina, M., Cicova, Z., Gela, D., Cosson, J., Nebesarova, J., Linhart, O., 2008. Morphology, chemical contents and physiology of chondrostean fish sperm: A comparative study between Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*). J. Appl. Ichthyol. 24, 371-377.

Psenicka, M., Alavi, S. M. H., Rodina, M., Gela, D., Nebesarova, J., Linhart, O., 2007. Morphology and ultrastructure of Siberian sturgeon, *Acipenser baerii*, spermatozoa using scanning and transmission electron microscopy. Biol.Cell 99 (2) 103-115.

Psenicka, M., Rodina, M., Nebesarova, J., Linhart, O., 2006. Ultrastructure of spermatozoa of tench *Tinca tinca* observed by means of scanning and transmission electron microscopy. Theriogenology, 66, 1355-1363.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. J. Fish Biol. 65, 319–332.

Rosenthal, H., Klumpp, D., Willfuehr, J., 1988. Infuence of sperm density and contact time on herring egg fertilization. J. Appl Ichthyol. 4, 79–86.

Rosengrave, P., Taylor, H., Montgomerie, R., Metcalf, V., McBride, K., Gemmell, N.J., 2009. Chemical composition of seminal and ovarian fluids of Chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. Com. Biochem. Physiol A. 152, 123–129.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234:1-28.

Scott, A.P., Baynes, S.M. 1980. A review of the biology, handling and storage of salmonid spermatozoa. J. Fish Biol. 17, 707–739.

Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnier, C., Fauvel, C., 1994. Sperm features in turbot (*Scophthalmus maximus*): a comparison with other freshwater and marine fish species. Aquat. Living Resour. 7, 283–294.

Suquet, M., Dreanno, C., Dorange, G., Normant, Y., Quemener, L., Gaignon, J.L., Billard, R., 1998. The ageing phenomenon of turbot, *Scophthalmus maximus*, spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization and storage capacities. J. Fish Biol. 32, 31–41.

Tuset, V.M., Trippel, E.A., de Monserrat, J., 2008. Sperm morphology and its influence on swimming speed in Atlantic cod. J. Appl. Ichthyol. 24 (2008), 398–405.

Tvedt, H.B., Benfey, T.J., Martin-Robichaud, D.J., Power, J., 2001. The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, Hippoglossus hippoglossus. Aquaculture, 194, 191–200.

Van Look, K.J.W., Kime, D.E., 2003. Automated sperm morphology analysis in fishes: the effect of mercury on goldfish sperm. J. Fish Biol. 63, 1020–1033.

Vladic, T.V., Jarvi, T., 2001. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. Proc. Soc. Lond. B Biol. Sci. 268, 2375–2381.

Wei, Q., Li, P., Psenicka, M., Alavi, S. M. H., Shen, L., Liu, J., Peknicova, J., Linhart, O., 2007. Ultrastructure and morphology of sperm in Chinese sturgeon, *Acipenser sinensis,* using scanning and transmission electron microscopy. Theriogenology, 67, 1269–1278.

Wojtczak, M., Glogowski, J., Koldras, M., Kucharczyk, D., Ciereszko, A., 2003. Charcterization of protease inhibitors of seminal plasma of cyprinids. Aquat. Living Resour. 16, 461–465.

Yaron, Z., 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129, 49–73.

English summary

In this study, sperm morphology, seminal plasma charcteristics and sperm motility and behaviour were studied in three teleostean fishes, *Barbus barbus* (Cyprinidae), *Perca fluviatilis* (Percidae), *Esox lucius* (Esociade) and one chondorstean fish, *Acipenser ruthenus* (Acipenseridae).

In all species, spermatozoon is composed of a head, a midpiece and a flagellum. Spermatozoon of teleostean species is without acrosome, while there is an acrosomal complex at tip of the head of *A. ruthenus*. Head shape is elongated in *A. ruthenus*, but ovoid or roundish in teleosten fishes. Nucleus vesicles were in *B. barbus*. Number of mitochondria highly differs among species, 4–6, 1 and 2–6 in *B. barbus*, *P. fluviatilis* and *A. ruthenus*, respectively. Flagellum has typical 9+2 pairs of microtubules with both dynein arms. In *A. ruthenus* and *E. lucius*, fin(s) were observed along the flagellum, which help sperm cells for moving during activation. Length of flagellum in *E. lucius* (30 µm) and *P. fluviatilis* (30–35 µm) was significantly shorter than *B. barbus* (47–54 µm) and *A. ruthenus* (43 µm). In *B. barbus*, the morphological and ultrastructural characteristics of spermatozoa differ during the reproductive season, may be due to aging phenomenon.

K^+ and Na^+ ions concentrations showed high variations among species as well osmolality. K^+ concentration was significantly higher in *B. barbus* (84 mM) compared to that of *E. lucius* (24.8 mM) and *P. fluviatilis* (11 mM). In contrast, Na^+ concentration in *B. barbus* (70–76 mM) was clearly lower; 131 and 116 mM in *P. fluviatilis* and *E. lucius*, respectively. Cl^- was 120–125, 107 and 116 mM in in *B. barbus*, *P. fluviatilis*, *E. lucius*, respectively. Na^+, K^+ and Cl^- concentrations were measured 20, 1 and 51 mM in *A. ruthenus*, respectively Osmolality were measured 268–276, 284, 284, and 51 mOsmol kg^{-1} in *B. barbus*, *P. fluviatilis*, *E. lucius* and *A. ruthenus*, respectively. The observed differences can be due to specific secretory activities of the male reproductive organs, especially sperm duct The ionic composition and osmolality of the seminal plasma did not change through the reproductive season in *B. barbus*. But, osmolality significantly decreased ain *P. fluviatilis* at the end of reproductive season.

Sperm volume in chondrostean is usually higher than teleostean fishes. It was decreased in *B. Barbus* towards the end of the reproductive season, but increased in *P. fluviatilis*. The highest and lowest spermatozoa concentration was observed in *P. fluviatilis* and *A. ruthenus* (29×10^9 ml^{-1} *vs.* 0.4×10^9 ml^{-1}). The spermatozoa concentration was $12–19 \times 10^9$ ml^{-1} and 23×10^9 ml^{-1} in *B. barbus* and *E. lucius*, respectively. Spermatozoa concentration was significantly decreased in *B. barbus* and *P. fluviatilis* towards the end of the reproductive season. The changes of sperm volume and spermatozoa concentration are due to endocrinological process regulating final sperm maturaion after realsing from testes to sperm duct.

Spermatozoa of these fishes was immotile in the seminal plasma and their motility was triggered in a hypo-osmotic condition. Sperm motility in the seminal plasma was inhibited due to high osmolality in teleostean fishes, but high K^+ concentration prevented sperm motility in the seminal plasma of *A. ruthenus*. Duration of sperm motility was very short in teleostean fishes (less than 1 min in distlled water) compared to *A. ruthenus* (longer than 2 min in distilled water). All parameters of sperm motility (spermatozoa velocity, percentage of motility and beat frequency) decreased rapidly after triggering of

sperm motility. These decreases are due to decrease of ATP content of spermatozoa, which occur rapidly after activation of sperm motility. The velocity of the spermatozoa vary among species. At 10–15 s post activation in distilled water, the spermatozoa velocity was measured 95–105 in *B. barbus*, 105–125 in *P. fluviatils*, 175–185 in *E. lucius* and 160–180 in *A. ruthenus*. Spermatozoa velocity depends on several parameters such as head dimension, beat frequency, length of flagellum, physical parameters of wave propagation such as wave length and amplitude. Higher spermatozoa velocity in *E. lucius* and *A. ruthenus* can be also due to presence of fin along the flagellum.

The optimum sperm motility parameters of *P. fluviatilis*, *E. lucius*, *B. barbus* and *A. ruthenus* were observed in osmolalities 100–200, 125–235, 200–235 and 25–50 mOsmol kg^{-1}, respectively. In *B. barbus*, *P. fluviatilis*, *E. lucius* and *A. ruthenus*, the motility of sperm was inhibited at 330–350, 300, 375, and 150–175 mOsmol kg^{-1}. Hypo-osmotic shock was observed to influence on flagellar structure during sperm motility. At 30 s post sperm activation, blebs appeared along the flagellum of spermatozoa of all teleostean species, which prevent correct and efficient wave propagation. The tip of the flagellum become curled into a loop shape which shorten the flagellum.

When sperm is observed a few second after activation, the waves propagte the whole length of the flagellum. A few second after activation, these waves are restrictaed to the part of the flagellum close to the head. Close to the end of sperm motility, only a very restricated portion of the flagellum in the vicinity of the head will exhibit wave. The ending process is the full absence of wave leading to immotility. Studies on *E. lucius* sperm showed that the number of waves and curvatures differed significantly in relation to osmolality of the activation media. At the same osmolality, wave length and amplitude were decreased, but number of curvatures and waves increased as a function of time post activation. Ca^{2+} ions infleunce on sperm motility through changes of flagellar waveforms and parameters. In these studies, flagellar beating was symmetricin teleostean fishes, but Ca^{2+} at high alkalinity induced assymetric behavior of sperm in *A. ruthenus*.

In contrast to teleostean species, *A. ruthenus* sperm exhibited an acrosin- like activity, which was significantly higher in sperm extract than seminal plasma. In samples of sperm acidic extract, total protease and serine activities were accumulated in protein band with relative molecular mass of 33kDa, which seems to be an acrosin. Protease inhibitors effects on protease activity of sturgeon sperm seems to be a species–specific and a dose dependent manner. Studies on *A. ruthenus* sperm reveals that high concentrations of protease inhibitors required for giving complete inhibition of sturgeon sperm motility. This study on *A. ruthenus* sperm showed that both trypsin- (AGB) and chymotrypsin- like (TPCK) inhibitors had significant effect on sperm motility in dose and time dependent manner.

Taken together, this study showed inter-family and inter-species differences in terms of morphology and ultrastructure of sperm, seminal plasma characteristics, and sperm motility and behaviour, which can be used for fish phylogeny and systematics.

Czech Summary

Tato práce pojednává o studiu morfologie spermií, charakteristikách semenné plazmy, motilitě a reakcích spermi tří druhů kostnatých ryb – parmy obecné (*Barbus barbus*, Cyprinidae), okouna říčního (*Perca fluviatilis*, Percidae) štiky obecné (*Esox lucius*, Esocidae) a jednoho zástupce chrupavčitých ryb - jesetera malého (*Acipenser ruthenus*, Acipenseridae).

U všech těchto druhů se spermie skládají z hlavičky, střední části a bičíku. Spermie kostnatách ryb jsou bez akrozómu, zatímco u jesetera malého je na vrcholu hlavičky spermie akrozomální komplex. Hlavička spermie jesetera malého je protáhlá, u kostnatých ryb je vejčitá či kulovitá. U parmy byly pozorovány nukleární vesikuly. Počet mitochondrií se velice liší mezi druhy: 4–6 u parmy, 1 u okouna a 2–6 u jesetera. Bičík má typickou strukturu 9+2 párů mikrotubulů s dyneinovými raménky. U spermií jesetera malého a štiky byla pozorovány „plutvičky“ (ploutvička) podél celého bičíku, které napomáhají pohybu spermie během její aktivace. Délka bičíku spermie štiky (30 μm) a okouna (30–35 μm) bya signifikatně kratší než u spermií parmy (47–52 μm)a jesetera malého (43 μm). U parmy se morfologické a ultrastrukturální charakteristiky lišily během reprodukčního období, pravděpodobně v důsledku fenomenu stárnutí.

Koncentrace iontů K^+ a Na^+ v semenné plazmě vykazovala velkou variabilitu mezi druhy , stejně tak osmolalita. Koncentrace K^+ byla signifikantně vyšší u parmy (84 mM) v porovnání se štikou (24,8 mM) a okounem (11 mM). Naopak, koncentrace Na^+ byla u parmy (70–76 mM) zřetelně nižší než u okouna(131 mM) a štiky (116 mM). U jesetera malého byla v semenné plazmě koncentrace iontů Na^+ 20 mM, K^+ 1mM, Cl^- 51mM. U parmy byly naměřeny hodnoty osmolality 268–276 $mOsmol.kg^{-1}$, u okouna 284 $mOsmol.kg^{-1}$, u štiky 284 $mOsmol\ kg^{-1}$ a 51 $mOsmol.kg^{-1}$ u jesetera malého. Pozorované rozdíly mohou být způsobeny specifickou sekreční aktivitou samčích reprodukčních orgánů, zvláště vývodných cest. Iontové složení a osmolalita semenné plazmy se neměnily během výtěrového období u parmy. U okouna na konci výtěrového období signifikantně poklesla osmolalita.

Objem speramtu u chrupavčitých ryb je obvykle vyšší než u ryb kostnatých. U parmy ke konci reprodukčního období klesal, u okouna naopak stoupal. Nejvyšší koncentrace spermií byla pozorována u spermatu okouna ($29 \times 10^9\ ml^{-1}$) , nejnižší u jesetera malého ($0{,}4 \times 10^9\ ml^{-1}$), u parmy byla koncentrace 12-19 $\times 10^9\ ml^{-1}$, u štiky $23 \times 10^9\ ml^{-1}$. Koncentrace spermií u parmy a okouna ke konci výtěrového období signifikantně klesala. Změny objemu spermatu a koncentrace spermií jsou způsobeny endokrinními procesy regulujícími filální dozávání spermií po jejich uvolnění z testes do vývodnách cest.

Spermie studovaných druhů byly imotilní v semenné plazmě a jejich pohyb byl spuštěn v hypoosmotickém prostředí. Pohyb spermie v semenné plazmě byl u kostnatých ryb inhibován vysokou osmolalitou, obsah K^+ předcháze pohybu spermií v semenné plazmě u jesetera malého. Doba pohybu spermií byla u kostnatých ryb velice krátká (méně než 1 min v destilované vodě) v porovnání s dobou pohybu spermií jesetera malého (více než 2 min v destilované vodě). Všechny parametry pohybu spermií (rychlost pohybu spermií, procento pohyblivých seprmií a frekvence pohybu bičíku) po aktivaci rychle klesají. Tento pokles je způsoben poklesem obsahem ATP ve spermiích,

který nastává rychle po aktivaci pohybu spermií. Rychlost pohybu spermií se liší mezi druhy. Rychlost pohybu spermií 10-15 s po aktivaci v destilované vodě byla 95–105 μm s^{-1} u parmy, 105–125 μm s^{-1} u okouna, 175–185 μm s^{-1} u štiky a 160–180 μm s^{-1} u jesetera malého. Rychlost spermií závisí na různých parametrech, jako je velikost klavičky, frekvenci pohybu bičíku, délce bičíku, parametrech bičíku jako jsou délka zvlnění bičíku a amplitudě výkyvu bičíku. Vyšší rychlost spermií štiky a jesetera malého může být také způsobena přítomností „ploutvičky" podél bičíku.

Optimální parametry motility spermií okouna, štiky, parmy a jesetera malého byly pozorovány v osmolalitách 100–200 (okoun), 125–235 (štika), 200–235 (parma) a 25–50 mOsmol kg^{-1} (jeseter malý). Inhibice pohybu spermií byla pozorována u parmy při osmolalitě 330–350 mOsmol kg^{-1}, u okouna při 300 mOsmol kg^{-1}, parmy při 375 mOsmol kg^{-1} a u jeseteramalého při osmolalitě 150–175 mOsmol kg^{-1}. Hypoosmotický šok ovlivňoval strukturu bičíku během pohybu spermie. 30 s po aktivaci spermií se u všech kostnatých ryb objevují na bičíku „puchýřky", které zabraňují správnému a účinnému vlnění bičíku. Zakončení bičíku se ohýbá do smyčky, která zkracuje bičík.

Pozorujeme-li sperma několik sekund po aktivaci, zvlnění bičíku se šíří po celé délce bičíku. Po několika sekundách od aktivace se vlnění bičíku omezuje jen na část bičíku blíže hlavičky. Ke konci pohybu spermií vykazuje vlnění pouze velice zkrácený úsek bičíku v oblasti přiléhající k hlavičce. Ukončení vlnění bičíku vede k ukončení pohybu spermie. Studie provedená na spermiích štiky ukázala, že se počet vln a ohybů bičíku různí v závislosti na osmolalitě aktivačního média. Při stejné osmolalitě se délka vlny a amplituda vlny zmenšuje, ale počet ohybů a vln narůstá v čase po aktivaci. Ionty Ca^{2+} ovlivňují pohyb spermií prostřednictvím změn parametrů zvlnění bičíku. V těchto studiích byl pohyb bičíku u kostnatých ryb symetrický, ale při vysokém pH vyvolal iont Ca^{2+} asymetrii pohybu spermií jesetera malého.

Oproti kostnatým druhům, vykazovalo sperma jesetera malého akrozinu podobnou aktivitu, která byla prokazatelně vyšší v extraktu spermií než v semenné plazmě. V okyselených vzorcích spermatu, byla celková aktivita proteázy a serinu akumulována v proteinových vazbách s relativní molekulární hmotností 33kDa, které vypadali jako akrosin. Efekt inhibitorů proteázy na aktivitu proteázy jeseterů je pravděpodobně druhově specifický a závisí na dávce (koncentaci). Studie na spermatu *A. ruthenus* naznačují, že pro kompletní inhibici pohybu jeseteřích spermií jsou požadovány vysoké koncentrace inhibitorů proteázy. Stejná studie na *A. ruthenus* prokázala, že trypsin- (AGB) a chymotripsin (TPCK) inhibitory měli signifikantní vliv na pohyblivost spermií v závislosti na dávce a čase.

Celkově vzato, tyto studie prokázaly rozdíly mezi čeleděmi a druhy v morfologii a ultrastruktuře spermií, ukazatelích semenné plasmy, pohybu a chování spermií, které mohou využity pro účely fylogeneze a systematiky.

Acknowledgements

I wish to express my deepest sense of gratitude to my supervisor, Prof. Otomar Linhart and my advisor Dr. Jacky Cosson for their encouragement and endless support throughout this study.

I will always be indebted to Dip.-Ing. Marek Rodina Ph.D. for teaching me laboratory techniques. I also wish to thank Assoc. Prof. Tomas Policar, Ph.D., Associ. Prof. Pavel Kozak, Ph.D. and Dipl.-Ing. David Gela, Ph.D. for letting me collaborate with them in several studies. During my study, I have been always involved in several studies, which the results have not been included in this thesis. I would like to thank all of my colleagues for letting me collaborate with them; Dr. Marc Legendre (IRD, Montpellier, France), Igor Babiak (Bodo University College, Bodo, Norway), Kazuo Inaba (Tsukuba University, Shimoda, Japan), Associ. Prof. Jana Peknicova, Pavla Manaskova-Postlerova, Ph.D., and Pavel Koubek Ph.D., (Institute of Biotechnology, AV CR, Prague, Czech Republic), Jana Neberasova and Martina Tesarova (Electron Microscopy Lab, JCU, Ceske Budejovice, Czech republic), Prof. Andrzej Ciereszko (Polish Academy of Sciences, Olstzyn, Poland), Associ. Prof. Martin Flajshans (VURH, Vodnany, Czech Republic).

I thank Ph.D. studentx at VURH for their kind helps and collaborations in parts of this study; Martin Psenicka, Vojtech Kaspar and Azadeh Hatef.

My heartiest thanks are due to Helena Linhartova and Monik Cosson for their kind attentions to me. Madam Marketa Flajshansova opened for me a new world with teaching Czech Language, I thank her in advances.

Technical assistance from the staff at the hatchery and aquaculture station is greatly acknowledged. I would like to thank all employee at VURH for their kind relationship during my study.

Last but not least, I thank my parents, brothers (Mahdi and Hossein) and sister (sedigheh) for their never ending encouragement. In fact, they warmly helped me to travel aborad and continue my research.

The PhD work was supported by:

1) Research plans of USB RIFCH No. MSM6007665809 financed by Ministry of Education, Youth and Sport of the Czech Republic (responsible leaders Prof. Dipl.-Ing. Otomar Linhart, DrSc.).
2) LC06073 - Centre for research of biodiversity (2006-2010, coordinator Institute of Systems Biology and Ecology of CAS in České Budějovice, leader for a part solved at USB RIFCH prof. Dipl.-Ing. Otomar Linhart DrSc.).
3) GA Project - 524/06/0817 Ultrastructure, Energetic and Competition in Spermatozoa: A Comparative Study Using Two Model Species of Chondrostean and Teleostean Fishes (2006-2008, investigator Prof. Dipl.-Ing. Otomar Linhart, DSc., Doc. RNDr. Jana Pěknicová, CSc.)
4) GA Project - 524/03/0178 A detailed study of spermatozoa in model species of chondrostean and teleostean fish (2003-2005, investigator Prof. Dipl.-Ing. Otomar Linhart, DSc.)
5) IAA608030801 Diversity of Bioenergetics Pathways, Membrane Functions, Signaling Mechanisms and Proteomics of Cryopreserved Sperm of Evolutionary Different Fish Species (2008-2012, investigator Prof. Dipl.-Ing. Otomar Linhart, DSc.)
6) GA523/09/1793 - The effect of endocrine disruptors on the reproduction parameters and gene expression in chosen gene of mouse and fish gonads (2009-2011, Biotechnological Institute of Czech Academy Sciences, responsible leader Doc. RNDr. Jana Pěknicová, CSc. and leader for a part solved at USB RIFCH Hadi Alavi, MSc.)

Publications

Book and book chapter

Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G., 2008. *Fish Spermatology.* Alpha Science Ltd, Oxford, UK. Pp. 465. ISBN 978-1-84265-369-2.

Alavi, S.M.H., Linhart O., Coward K., Rodina M., 2008. Fish spermatology: Implication for aquaculture management. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, R. (Eds.). pp. 397-460. Alpha Science Ltd, Oxford, UK.

Special issue for peer-reviewed journal

Alavi, S.M.H., Linhart, O., Rosenthal, H., 2008. *The proceedings of the First International Workshop on the Biology of Fish Sperm*. Journal of Applied Ichthyology, 24: 357 – 525.

Peer-reviewed journals

Imanpoor, M.R., **Alavi, S.M.H.,** Cosson, J., 2009. Relationship between biological characteristics of eggs and female brood stocks of Persian sturgeon, *Acipenser persicus*; a comparision with other fish species. Fish Physiology and Biochemistry. In press.

Alavi, S.M.H., Rodina, M., Policar, T., Linhart, O., Relationships between semen characteristics and body size in *Barbus barbus* L. (Teleostei: Cyprinidae) and effects of ions and osmolality on sperm motility. Comparative Biochemistry and Physiology – Part A. In press.

Alavi, S.M.H., Pšenička, M., Policar, T., Rodina, M., Hamáčková, J., Kozák, P., Linhart, O., Sperm quality in male *Barbus barbus* L. fed different diets during the spawning season. Fish Physiology and Biochemistry. In press

Alavi, S.M.H., Rodina, M., Viveiros, A.T.M., Cosson, J., Gela, D., Boryshpolets, S., Linhart, O., 2009. Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius* L.). Theriogenology. In press

Hatef, A., Niksirat, H., **Alavi, S.M.H.,** 2009. Composition of ovarian fluid in endangered Caspian brown trout, *Salmo trutta caspius*, and its effects on spermatozoa motility and fertilizing ability compared to freshwater and a saline medium. Fish Physiology and Biochemsitry. In press.

Psenicka, M., Cosson, J., **Alavi, S.M.H.,** Rodina, M., Kaspar, V., Gela, D., Linhart, O., Ciereszko, A., 2008. Staining of sturgeon spermatozoa with trypsin inhibitor from soybean, Alexa Fluor® 488 conjugate for visualization of sturgeon acrosome. Journal of Applied Ichthyology, 24:514-516.

Linhart, O., **Alavi, S.M.H.,** Rodina, M., Gela, D., Cosson, J., 2008. Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (*Cyprinus carpio*). Journal of Applied Ichthyology, 24:386-392.

Psenicka, M., **Alavi, S.M.H.,** Rodina, M., Cicova, Z., Gela, D., Cosson, J., Nebesarova, J., Linhart, O., 2008. Morphology, chemical contents and physiology of chondrostean fish sperm: A comparative study between Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*). Journal of Applied Ichthyology, 24:371-377.

Alavi, S.M.H., Psenicka, M., Policar, T., Linhart, O., 2008. Morphology and fine structure of *Barbus barbus* (Teleostei: Cyprinidae) spermatozoa. Journal of Applied Ichthyology, 24:378-381.

Yeganeh, S., Mojazi Amiri, B., **Alavi, S.M.H., 2008.** Motility of Mugil cephalus L. spermatozoa in coelomic fluid, seminal fluid and saline media. Journal of Applied Ichthyology, 24: 517-518.

Hulak, M., Rodina, M., **Alavi, S.M.H.,** Linhart, O., 2008. Evaluation of semen and urine of pike (*Esox lucius* L.): Ionic compositions and osmolality of the seminal plasma and sperm volume, density and motility. Cybium. 32(2): 189 – 190.

Legendre, M., Cosson, J., **Alavi, S.M.H.,** Linhart, O., 2008. Sperm motility activation in the euryhaline tilapia *Sarotherodon melanotheron heudelotii* (Dumeril, 1859) acclimatized to fresh, sea or hypersaline waters. Cybium, 32(2): 181 – 182.

Linhart, O., **Alavi, S.M.H.,** Rodina, M., Gela, D., Cosson, J., 2008. After finishing of motility, common carp (*Cyprinus carpio*) sperm is able to re-initiate a second motility period and to fertilize eggs. Cybium, 32(2): 187 – 188.

Alavi, S.M.H., Rodina, M., Policar, T., Cosson, J., Kozak, P., Psenicak, M., Linhart, O., 2008. Physiology and behavior of stripped and testicular sperm in *Perca fluviatilis* L. 1758. Cybium. 32(2): 162 – 163.

Alavi, S.M.H., Psenicka, M., Policar, T., Rodina, M., Kozak, P., Linhart, O., 2008. Sperm characteristics in *Barbus barbus* as a function of nutrition throughout the reproductive season. Cybium, 32(2): 200-201.

Alavi, S.M.H., Rodina, M., Cosson, J., Psenicka, M., Linhart, O., 2008. Roles of extracellular Ca2+ and pH on motility and flagellar wave form parameters in sturgeon spermatozoa. Cybium. 32(2): 124 – 126.

Alavi, S.M.H., Psenicka, M., Rodina, M., Policar, T., Linhart, O., 2008. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Cyprinidae: Teleostei) during the reproductive season. Aquatic Living Resources, 21, 75 - 80.

Kaspar, V., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Kocour, M., **Alavi, S.M.H.,** Hulak, M., Linhart, O., 2006. Equalizing sperm concentrations in a common carp (*Cyprinus carpio*) sperm pool does not affect variance in proportions of larvae sired in competition. Aquaculture, 272S1: S204 – S209.

Hatef, A., Niksirat, H., Mojazi Amiri, B., **Alavi, S.M.H.,** Karami, M., 2007. Sperm density, seminal plasma composition and their physiological relationship in the endangered Caspian brown trout (*Salmo trutta caspius*). Aquaculture Research, 38: 1175 – 1181.

Alavi, S.M.H., Rodina, M., Policar, T., Kozak, P., Psenicka, M., Linhart, O. 2007. Semen of *Perca fluviatilis* L: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology, 68: 276-283.

Wei, Q., Li, P., Psenicka, M., **Alavi, S.M.H.,** Shen, L., Liu, J., Peknicova, J., Linhart, O., Ultrastructure and morphology of sperm in Chinese sturgeon (*Acipenser sinensis* Gray 1835) using scanning and transmission electron microscopy. Theriogenology, 67: 1269-1278.

Rodina, M., Gela, D., Kocour, M., **Alavi, S.M.H.,** Hulak, M., Linhart, O., 2007. Cryopreservation of tench, *Tinca tinca*, Sperm: Sperm motility and hatching success of embryos. Theriogenology, 67: 931-940.

Psenicka M., **Alavi, S.M.H.,** Nebesarova, J., Linhart O., 2007. Morphology and ultrastructure of Siberian sturgeon, *Acipenser baerii*, spermatozoa using scanning and transmission electron microscopy. Biology of the Cell, 99(2): 103-115.

Alavi, S.M.H., Cosson, J., Kazemi, R., 2006. Semen characteristics in *Acipenser persicus* in relation to sequential stripping. J. Applied Ichthyology, 22 (Suppl. 1): 400-405

Alavi, S.M.H., Cosson, J. 2006. Sperm motility in fishes: (II) Effects of ions and osmotic pressure. Cell Biology International, 30: 1-14

Alavi, S.M.H., Mojazi Amiri, B., Cosson, J., Karami, M., Abdoulhay, H., Pourkazemi, M., Akhoundzadeh, M.A. 2006. Determination of semen quality of the Persian sturgeon by seminal plasma parameters and sperm motility. Iranian J. Fisheries Sciences, 5(2): 1-18

Alavi, S.M.H., Cosson, J. 2005. Sperm motility and fertilizing ability in the Persian sturgeon, *Acipenser persicus*. Aquaculture Research, 36: 841-850

Alavi, S.M.H., Cosson, J. 2005. Sperm motility in fishes: (I) Effects of pH and temperature. Cell Biology International, 29: 101-110

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., Akhoundzadeh, M.A. 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: Effects of pH, dilution rate, ions and osmolality. Reproduction, 128: 819-828

Alavi, S.M.H., Cosson, J., Karami, M., Abdoulhay, H., Mojazi Amiri, B. 2004. Chemical composition and osmolality of seminal fluid of *Acipenser persicus*; their physiological relationship with sperm motility. Aquaculture Research, 35: 1238-1243

Selected Abstracts in conferences and symposiums

Alavi, S.M.H., Legendre, M., Cosson, J., Linhart, O., 2008. Effects of hypo- and hyper-osmotic shocks on biological performances of sperm in the euryhaline tilapia (*Sarotheron melanotheron heudelotii*, Dumeril, 1859) acclimatized to fresh, sea and haypersaline waters. *The 1st International Workshop on Aquatic Toxicology and Bio monitoring*, Vodnany, Czech Republic. 27 – 29 August, 2008. P. 80.

Alavi, S.M.H., Cosson, J.J., Rodina, M., Gela, D., Linhart, O., 2008. Traits of sperm motility in freshwater fishes. Biotechnology, 2008. University of South Bohemia, 13 – 14 February.

Alavi, S.M.H., Cosson, J., Legendre, M., Rodina, M., Linhart, O., 2008. A review on sperm motility characteristics in freshwater fish. Aquaculture Europe 2008, Krakow, Septemebr 15 – 18, 2008. Pp. 256 – 257.

Alavi, S.M.H., Psenicka, M., Policar, T., Hamackova, J., Rodina, M., Linhart, O., 2007. Ultrastructure, biochemical characteristics and motility of *Barbus barbus*

spermatozoa. In: The 1st International Workshop on the Biology of Fish Sperm, Vodnany, Czech Republic, 29-31 August, 2007. Pp. 10 – 11.

Alavi, S.M.H., Rodina, M., Cosson, J., Linhart, O., 2007. Roles of extracellular Ca^{2+} and pH on motility and flagellar waveform parameters in sturgeon spermatozoa. In: The 8th International Symposium in Reproductive Physiology of Fish. June 3-8, Saint-Malo, France. P. 46.

Alavi, S.M.H., Rodina, M., Policar, T., Cosso, J., Kozak, P., Psenicka, M., Linhart, O., 2007. Physiology and behavior of stripped and testicular sperm in *Perca fluviatilis* L. 1758. In: The 8th International Symposium in Reproductive Physiology of Fish. June 3-8, Saint-Malo, France. P. 175.

Alavi, S.M.H., Psenicka, M., Policar, T., Rodina, M., Kozak, P., Linhart, O., 2007. Sperm characteristics in *Barbus barbus* as a function of nutrition throughout the reproductive season. In: The 8th International Symposium in Reproductive Physiology of Fish. June 3-8, Saint-Malo, France. P. 215.

Legendre, M., Cosson, J., **Alavi, S.M.H.,** Linhart, O., 2007. Sperm motility activation in the euryhaline tilapia *Sarotherodon melanotheron heudelotii* (Dumeril, 1859) acclimatized to fresh, sea or hypersaline waters. In: The 8th International Symposium in Reproductive Physiology of Fish. June 3-8, Saint-Malo, France. P. 192.

Hulak, M., Rodina, M., **Alavi, S.M.H.,** Linhart, O., 2007. Evaluation of semen and urine of pike (*Esox lucius* L.): Ionic compositions and osmolality of the seminal plasma and sperm volume, density and motility. In: The 8th International Symposium in Reproductive Physiology of Fish. June 3-8, Saint-Malo, France. P. 197.

Linhart, O., **Alavi, S.M.H.,** Psenicka, M., Rodina, M., Cosson, J., Mims, S.D., Shelton, W.L., 2006. Structure, motility and cryopreservation of sperm of chondrostean fish species, A review. The 10th International Symposium on Spermatology. Madrid, 17-22 September 2006, Spain.

Alavi, S.M.H., Rodina, M., Psenicka, M., Gela, D., Flajshans, M., Kocour, M., Policar, T., Linhart, O., Cosson, J., 2006. Spermatology as a tool in aquaculture management. World Congress SVU 2006, Českých Budějovicích, 26-30 June, Czech Republic

Alavi, S.M.H., Cosson, J., Karami, M., Mojazi Amiri, B., 2005. Effects of stripping frequency on composition of seminal plasma and sperm density and motility in the Persian sturgeon, *Acipenser persicus.* In: The 5th International Symposium on Sturgeon, May 9-13, Rasht, Iran. Pp: 13-17.

Karami, M., **Alavi, S.M.H.,** 2005. Some biological aspects of *Acipenser persicus* in the southern part of the Caspian Sea: (II) Age, length and weight relationships. In: The 5th International Symposium on Sturgeon, May 9-13, Rasht, Iran. Pp: 187-189.

Rafiee, R., **Alavi, S.M.H.,** Karami, M., Mirvaghefi, A.R., 2005. Some biological aspects of *Acipenser persicus* in the southern part of the Caspian Sea: (I) Morphometric and meristic characteristics. In: The 5th International Symposium on Sturgeon, May 9-13, Rasht, Iran. Pp: 197-200.

Training and Supervision Plan during Study

Name	Sayyed Mohammad Hadi Alavi
Laboratory	Reproductive Physiology in Fish
Daily supervisor	Pro. Dipl.-Ing. Otomar Linhart
Supervisor	Prof. Dipl.-Ing. Otomar Linhart, DSc.
Period	3th October 2005 until 24th of September 2009

PhD courses	**year**
Fish reproduction	2006
Applied Hydrobiology	2007
Aquatic Toxicology	2007
Biostatistics	2007
English Language	2007

Scientific seminars	**year**
Seminar days of RIFCH	2006 2007 2008 2009

International conferences	**Year**
1- 8th International Symposium on Reproductive Physiology of Fish. June 3–8, St. Malo, France (*Oral presentation and posters*).	2007
2- 1st International Workshop on Biology if Fish Sperm, August 29-31, Vodnany, Czech Republic (*Oral presentation and posters*).	2007
3- The 1st International Workshop on Aquatic Toxicology and Biomonitoring, Vodnany, Czech Republic. 27 – 29 August (*Posters*)	2008
4- European Aquaculture Conference, Krakow, Poland, September 15-18 (*oral presentation*)	2008
National conference	
Biotechnologie, Jicoceska Univerzita, ceske Budejovice, unora 13 – 14 *(Poster presentation)*	

Foreign stays during PhD study at RIFCH	**year**
Dr. Marc Legendre, Ph.D., IRD/GAMET, B.P. 5095, 34033 Montpellier, France *Duration of Stay:* 1 month, Research topic: Mechanism of sperm motility in fish	2007 2008
Prof. Igor Babiak, Ph.D., Bodo University College, 8049 Bodo, Norway *Duration of stay:* 1 month, *Research topic:* Biology of sperm in marine fishes	2008 2009

Curriculum Vitae

Surname: Alavi
First name: Sayyed Mohammad Hadi
Title: M.Sc.
Date of Birth: 17th September, 1978, Tehran, Iran
Nationality: Iranian
Address: Research Institute of fish Culture and Hydrobiology, University of South Bohemia (USB), 389 25 Vodňany, Czech Republic. Tel.: +420 387 77 4610, fax: +420 383 382 396, e-mail: alavi@vurh.jcu.cz

Education

Sept. 1996 – Sept. 2000: B.Sc. in Fisheries Science, University of Tehran, Iran
Sept. 2000 – March 2003: M.Sc. in Fisheries Science, University of Tehran, Iran
Oct. 2005 – Sept 2009: Ph.D. student at the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Laboratory of Reproductive Physiology in Fish.

Research interests

- Reproductive Physiology in Fish
  - Sperm maturation
  - Sperm morphology
  - Sperm physiology and biochemistry
  - Motility and energetics of sperm
  - Mechanism of fertilization in fish
  - Acrosoma reaction in sperm
  -
- Ecotoxicology
  - Effects of endocrine disruptors on male reproductive physiology

Experiences

Fish reproduction
Cryopreservation of sperm
Electron microscopy