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Note

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

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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 spermatozoa 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 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 degradation 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

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osmolality and ionic composition of the seminal plasma during the reproductive season.

2 Materials and methods

2.1 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^{TM} 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 °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 °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.

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

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

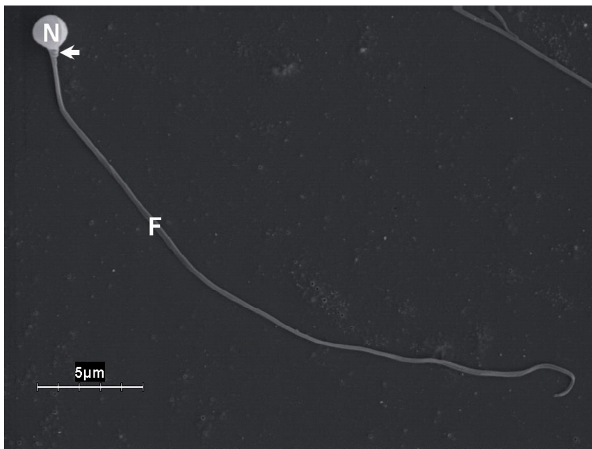

Fig. 1. Structure of *B. barbus* spermatozoa using scanning electron microscopy. Nucleus (N), Midpiece (arrow) and flagellum (F).

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

	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]
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[a]
	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]

though. The shortest and longest spermatozoa (both flagellar and total) were observed in March and April respectively.

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

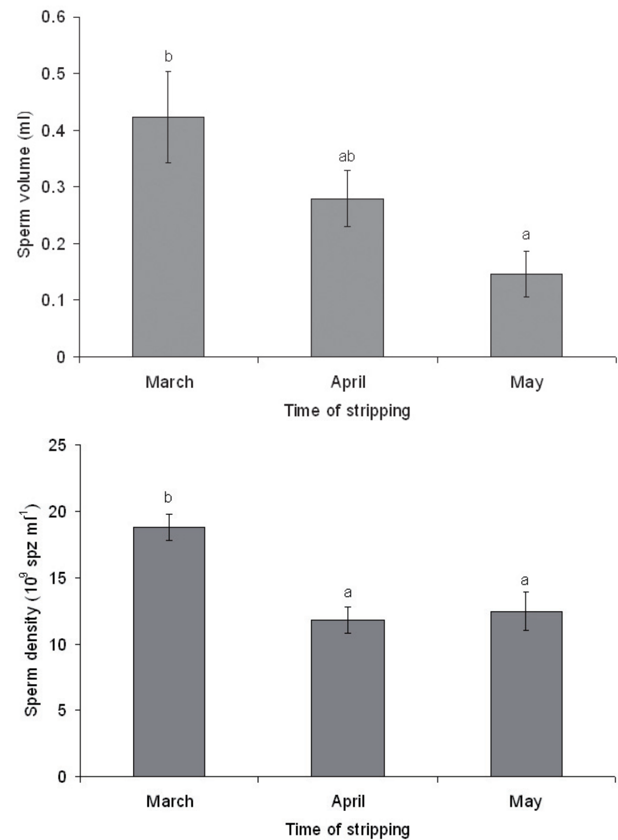

Fig. 2. Changes in sperm volume (a), and sperm density (b) in *B. barbus* during the reproductive season. Bars with different superscripts indicate statistically significant differences among stripping times ($p < 0.05$).

(Fig. 2b). However, no significant difference was observed between April and May.

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}).

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.

4 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

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, n.d.: not determined.

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.

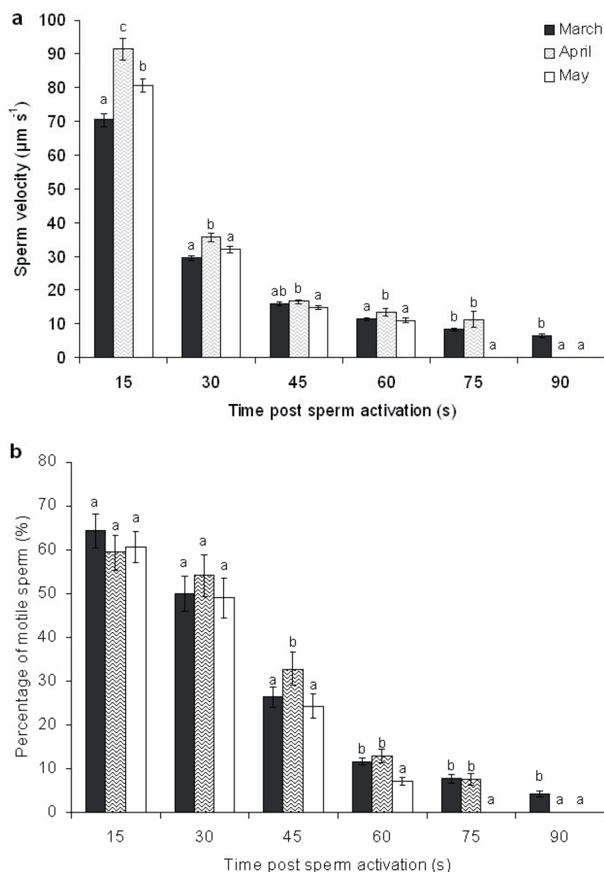

Fig. 3. Changes in sperm motility parameters: spermatozoa velocity (a) and percentage of motile spermatozoa (b) in *B. barbus* during the reproductive season. The velocity values correspond to motile sperm cells. Bars with different superscripts indicate statistically significant differences among strippings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

to bring about a diversity of changes in other fish species, affecting morphology, seminal plasma composition, sperm density, spermatozoa 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).

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.

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