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

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ABSTRACT: Dynamics of sperm motility (sperm velocity, percentage of motility and flagellar beat frequency) and monthly variations of semen characteristics (semen volume and osmolality and sperm concentration and motility) were studied in *Perca fluviatilis*. This study showed that sperm velocity, percentage of motility and flagellar beat frequency significantly and rapidly decreased after the activation of sperm motility. Twelve spermiating males were randomly selected and electronically tagged to study monthly variations of semen characteristics. The 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 it significantly increased in April. Sperm concentration was higher in November and January than in February and April. The highest and the lowest osmolality of semen was observed in January and April while it decreased in February and April. At 15 s post activation, the lowest percentage of motile spermatozoa was observed in November. The semen samples collected in April showed the lowest motility of spermatozoa (24.3%) at 30 s post activation. But the percentage of motile spermatozoa collected from November to February showed more than 65% motility at 30 s post activation. At the end of motility period (60 s post activation), no motile spermatozoa were observed in April, and the highest motility was in November. Sperm velocity did not show any significant differences at 15 s post activation. The lowest sperm velocity was observed in April at 30 s post activation.

Keywords: beat frequency; semen volume; sperm concentration; osmolality; sperm motility

Percid fish are candidates for commercial aquaculture because of their economic values as 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 fish is characteristic of annual spawner with group-synchronous development of gonads (Craig, 2000). *Perca* spawn once a year in spring. This annual rhythm 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, the perch needs a minimum 160-day chilling period at a temperature lower than 8°C. The males become mature at 2 years of age (Craig, 2000). Artificial propagations using different hormones were studied (Kouřil et al., 1997; Policar et al., 2008a).

Supported by Grand Agency of the Czech Republic (Project No. 523/09/1793), Ministry of Education, Youth and Sports of the Czech Republic (Project No. MSM 6007665809) and Ministry of Agriculture of the Czech Republic (Grant Nos. QH 71305, QH 91310 and IAA608030801).

The reproductive cycle and sex steroid profiles have already been described in males of 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 a key role in early spermatogenesis and spermiogenesis, however it is not declared if 17α, 20β-dihydroxy-4-pregnen-3-one is involved in spermiation (Nagahama, 1994; Miura and Miura, 2003; Vizziano et al., 2008).

It has already been 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 the reproductive season, both 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) and marine fish (*Melanogrammus aeglefinus*, Rideout et al., 2004; *Hippoglossus hippoglossus*, Babiak et al., 2006; *Gadus morhua*, Rouxel et al., 2008). 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 a triggering of the initiation of sperm motility (Cosson et al., 1999; Morisawa et al., 1999, 2008). Sperm velocity, percentage of motility and flagellar beat frequency are the main parameters considered in sperm quality assessment (Alavi et al., 2008a; Cosson, 2008).

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 was very variable in perch ranging from 0.55 ml to 6.78 ml; (b) the sperm concentration was in the range of 3.3–43.9 $\times 10^9$ spz/ml (mean: 29 $\times$ 10^9 spz/ml); (c) the motility duration of spermatozoa was very short (less than 1 min); (d) osmolality of the activation medium affected sperm motility parameters (sperm motility totally suppressed in the activation medium with osmolality higher than 300 mOsmol per kg); (e) the means of seminal plasma osmolality (mOsmol/kg), sodium, chloride, potassium and calcium concentrations (mM) were measured as 298, 131, 107, 11 and 2.4, respectively.

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

MATERIAL AND METHODS

Broodfish and sperm collection

To study the dynamics of sperm motility parameters, sperm of 4 spermiating males (total length 198–214 mm, body weight 89–117 g) with initial motility higher than > 80% were used. In the case of monthly variations of semen characteristics, twelve spermiating males of *P. fluviatilis* with total length ranging between 14 and 19 mm and weight of 49 to 83 g were randomly selected from a pond at the beginning of November and then transferred and kept in a tank in indoor conditions. These individuals were electronically tagged for use in this study. The tank of broodfish received a water flow of 10 l per min, and environmental conditions were controlled at: water temperature 21.5 ± 0.5°C, oxygen 6.8 ± 0.3 mg O_2/l 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 the abdomen and by means of a syringe with a needle. No hormone was used for the induction of spermiation (Policar et al., 2008b). 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.

Determinations of semen volume and osmolality and sperm concentration

Semen volume and sperm concentration were measured by weighting and counting, respectively, and according to methods described by Alavi et al. (2007). The semen volume and sperm concentration were expressed as ml and billion of spermatozoa per ml of semen, respectively. Semen was diluted 1 000 times in saline physiological solution. A Bürker cells haemocytometer counting chamber was used to count the sperm cells under a light microscope (200×). A droplet (10 µl) of 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 a low volume of semen to get enough seminal plasma.

Sperm motility assessments

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

Dynamics of sperm motility parameters

A two-step method was used to evaluate sperm motility in this study (Billard and Cosson, 1992). Firstly, the semen was diluted in an immobilizing solution (IS) containing NaCl 200mM, $NaHCO_3$ 2.38mM (osmolality 380 mOsmol/kg) at a 1:50 ratio. Right after predilution the sperm motility was triggered in an activation medium containing NaCl or KCl 50–100mM, Tris 20mM, pH 8.5 ± 0.5. Measurements of flagellar beat frequency were done 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. Before each experiment the frequency of the flash illuminator was adjusted to the highest previously observed value of flagellar frequency (around 60 Hz). 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.

Monthly variation of sperm motility parameters

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

Data analysis

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

RESULTS

Dynamics of sperm motility parameters

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

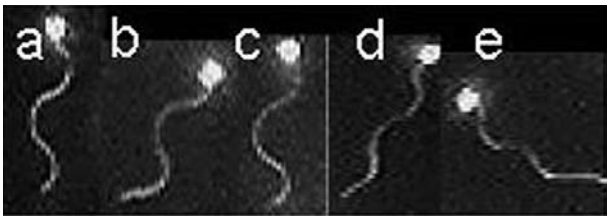

Figure 1. Motility of spermatozoa of *Perca fluviatilis* after activation in a medium with 100 mOsmol/kg at 10(a), 15(b), 35(c), 45(d) and 60(e) s post activation

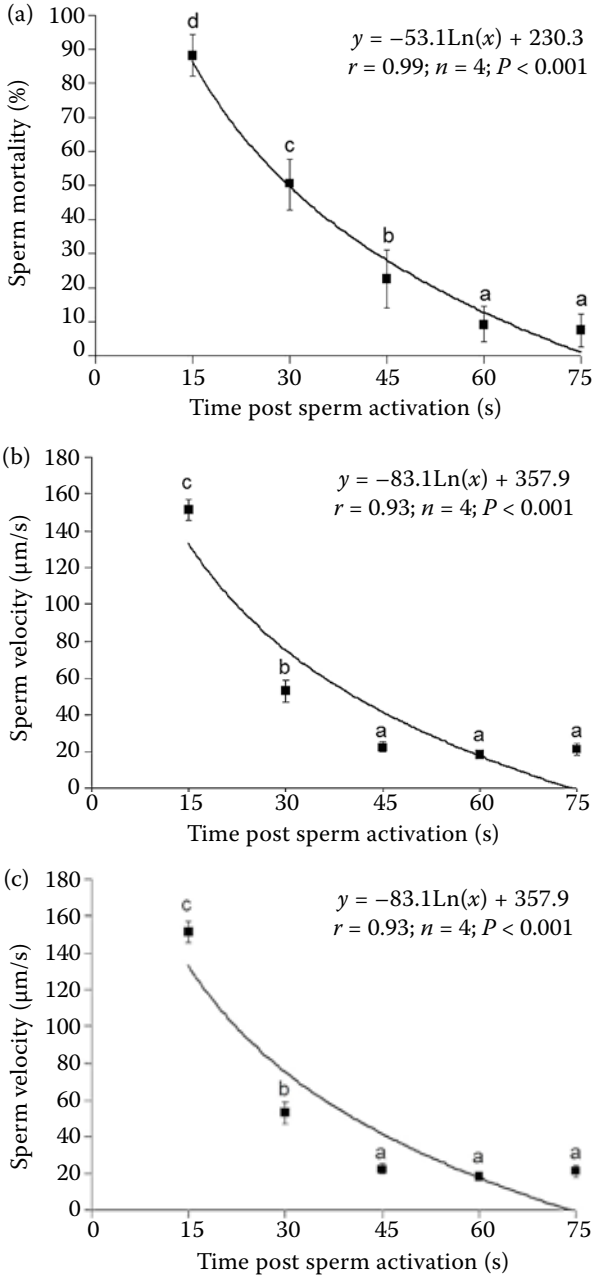

Figure 2. Percentage of sperm motility (a), sperm velocity (b) and flagellar beat frequency (c) in *Perca fluviatilis* after activation

Monthly variations of semen characteristics

Semen volume did not change significantly from November to February, but significantly increased in April (Table 1). Sperm concentration was higher in November and January than in February and April (Table 1). In contrast, the total number of spermatozoa (sperm concentration × semen volume) showed a 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).

Both the percentage of motile spermatozoa and sperm velocity decreased rapidly as a function of time after activation in all sampling times (Figure 3). The sampling time affected the percentage of motile spermatozoa significantly ($P < 0.01$). At 15 s post activation, the lowest percentage of motile spermatozoa was observed in November (Figure 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 was 94.6 and 24.3% at 15 and 30 s post activation). But the percentage of motile spermatozoa collected from November to February showed more than 65% motility at 30 s post activation. At the end of motility period (60 s post activation), no motile spermatozoa were observed in April, and the highest motility was in November. Sperm velocity did not show any significant differences at 15 s post activation (Figure 3b). Again, the lowest sperm velocity was observed in April at 30 s post activation. Sperm velocity highly decreased from 15 to 30 s post activation (from >150 µm/s to >14 µm/s, respectively), but the differences were low at 30 s post activation (9.7 µm/s and 5.8 µm/s at 45 and 60 s post activation, respectively).

DISCUSSION

Dynamics of sperm motility parameters

A decrease in motility parameters of fish spermatozoa has already been demonstrated in several species (see the review by Cosson et al., 1999; Alavi and Cosson, 2006). The beat frequency represents the number of waves generated each second. It is directly proportionate to the activity of dyneins and therefore to 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; 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) is lower than in pike (*Esox lucius*, 210 µm/s) (Alavi et al., 2009), Siberian sturgeon (*Acipenser baerii*, 200 µm/s) (Alavi et al., 2009), but higher than in carp (*Cyprinus carpio*, 150 µm/s) (Linhart et al., 2008) and

Table 1. Monthly variations of sperm volume, sperm 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's test)

Parameter	29th November 05	10th January 06	21st February 06	7th April 06
Sperm volume	0.12 ± 0.03^{a}	0.32 ± 0.07^{a}	0.07 ± 0.02^{a}	0.85 ± 0.23^{b}
Sperm 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 ± 9.05^{a}

tench (*Tinca tinca*, 160 µm/s) (Rodina et al., 2004). This is may be due to differences in sperm morphology (with emphasis on the head shape and flagellar length) and ATP content (Alavi et al., 2009).

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

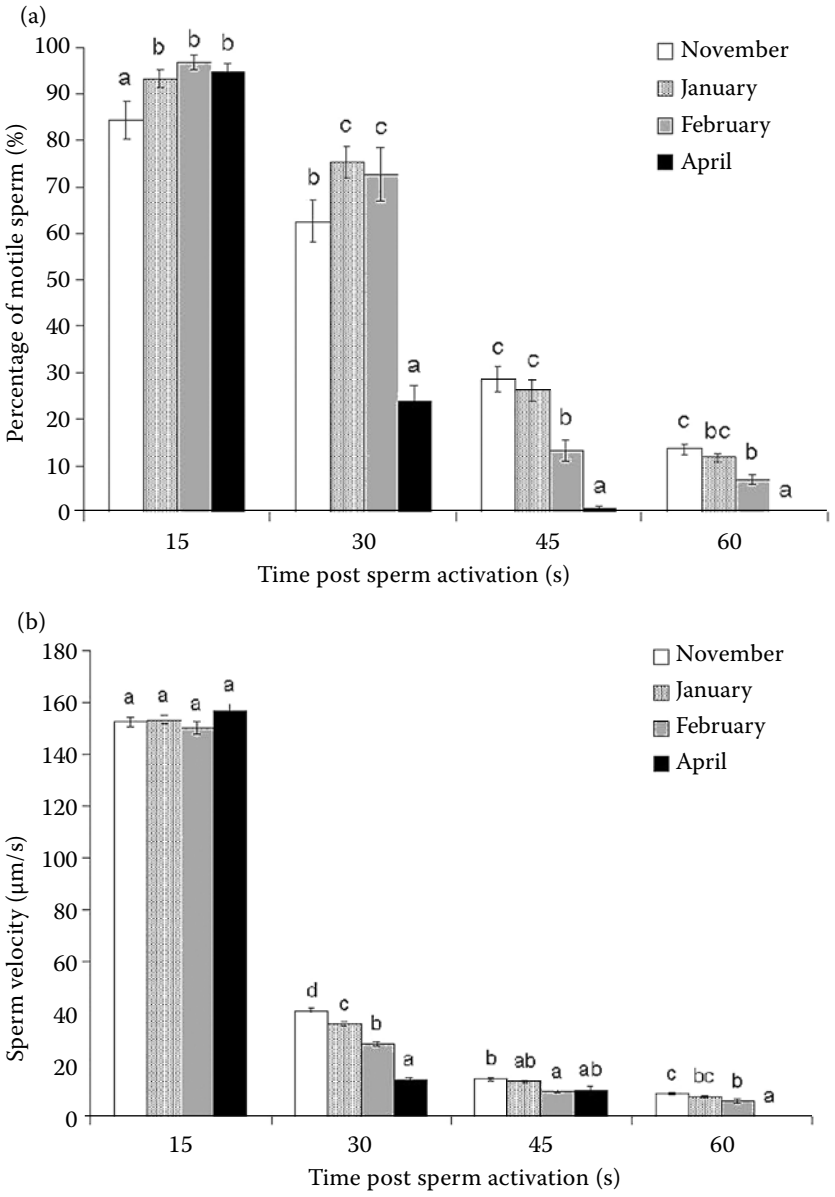

Figure 3. Monthly changes in the percentage of motile spermatozoa (%, a) and sperm velocity (µm/s, b) in *Perca fluviatilis*. Motility of prediluted sperm in NaCl 200mM, $NaHCO_3$ 2.38mM) (osmolality 380 mOsmol/kg) was activated in 30mM NaCl containing BSA 0.1%. Values represent the mean ± SE from 12 males with three replications per male. Values with the same superscript are not significantly different at the same time post activation ($P > 0.05$, Duncan's test)

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

Similarly to this study, a decrease in sperm 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), and rainbow trout (Munkittrick and Moccia, 1987). But it showed an increase in some species 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" phenomenon (hydration of semen), which showed high variation among individuals (Morisawa et al., 1979; Koldras et al., 1996; Alavi et al., 2008a,b). A decrease in semen osmolality from the beginning of spermiation (November) to the end (April) is related to hydration of semen that showed an increase in this study (semen volume was significantly higher in April). This is in contrast to changes in the osmolality of halibut sperm that showed a 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 in the middle of reproductive season, and was the 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, which 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 spermiation period). It has already been claimed that the percentage of sperm motility decreased as the reproductive season progressed (for example 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 a short-term storage through the spermiation period. Rouxel et al. (2008) found that the percentage of sperm motility of cod peaked in the middle part of the reproductive season after a short-term storage. Our previous study showed that osmolality affects the percentage of sperm motility of *P. fluviatilis* (the optimum sperm motility as observed in 100 mOsmol/kg). This finding can help us to establish a better activation medium for perch spermatozoa at the end of spermiation period (April) by adding Ca^{2+} to the activation medium or by increasing the osmolality. Sperm velocity did not change among sampling times at 15 s post activation, but it rapidly decreased at 30 s post activation (the highest decrease was observed in April). On the other hand, the velocity of spermatozoa in *P. fluviatilis* similar to that of Atlantic halibut (Babiak et al., 2006), cod (Rouxel et al., 2008) and common barbel (Alavi et al., 2008) decreased as the spermiation period progressed. This is due to an ATP decrease which is necessary for sperm movement. At 30 s post activation, the highest and the lowest sperm velocity was observed in November and April. It has already been demonstrated that longer spermatozoa swim faster, in *B. barbus* (Alavi et al., 2008b)

and *Salmo salar* (Vladic et al., 2002), due to more ATP available for axonemal beating (Vladic et al., 2002). Taken together, a decrease in sperm motility towards the end of spermiation period (either percentage of motility or velocity) probably results from the aging process which includes changes in sperm morphological parameters (flagellar length) and ATP contents.

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

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Received: 2009–05–05
Accepted after corrections: 2009–09–29

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