Sperm motility parameters of *Barbus barbus callensis* throughout the reproduction season: Computer aided semen analysis and gametes motility duration

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Abstract

The present study investigated the existing relationship between computer assisted semen motility parameters and gametes motility duration, known to express semen quality and fertilizing capacity in fish. The objective was particularly to identify computer aided semen analysis (CASA) parameters that could be used as potential fertility predictors. Semen samples were collected from the beginning to the end of the spawning season of *Barbus barbus callensis*, a freshwater fish abundantly distributed throughout North Africa. Semen was simultaneously analyzed using optical microscopy including semen motility duration (SMD) measurement, by the aid of a computer-assisted semen analyzer. The measured CASA parameters were: straight line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), linearity and straightness. The results showed that motility duration evolved in a similar manner as several CASA parameters. Significant correlation coefficients expressed these relationships with r = 0.74, 0.32, 0.16 and 0.45 for VSL, VAP, VCL and BCF, respectively. No correlations were observed when studying relationships between motility duration, STR, LIN and ALH, with r = 0.08, 0.06 and 0.006, respectively. The present results showed that CASA motility parameters are strongly related to motility duration. VSL was revealed as the main parameter being highly correlated to motility duration (r = 0.74). This quantitatively and objectively measured parameter is revealed to be a useful indicator of semen quality and could serve as a potential indicator of fertility outputs in fish.

Keywords: *Barbus barbus callensis*, Reproduction season, Semen motility, CASA.

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Introduction

The barbel (*Barbus barbus callensis*) is a freshwater fish inhabiting North Africa (Almaça, 1970). Although, this species has been reported in literature since 1842 (Cuvier and Valenciennes, 1842), little is known concerning the reproduction physiology, particularly concerning variations of semen motility parameters during the reproduction season.

The spermatozoa motility of most teleost fish differs from mammals. Gametes are immotile on ejaculation and the activation is induced only after the delivery of sperm into an aqueous environment or into artificial media (Scott and Baynes, 1980; Perchec-poupard *et al*., 1997; Kime *et al*., 2001). In freshwater species, spermatozoa are immotile in relation to the iso-Mackie Building osmolarity of the seminal fluid and a hypo-osmotic shock is required for the initiation of sperm motility (Kime *et al*., 2001).

Sperm quality, a key factor determining the fertilizing outputs, is significantly related to motility quality (Billard, 1978; Cosson *et al*., 1991; Lahnsteiner *et al*., 1997; Fauvel *et al*., 1999; Au *et al*., 2002). In fish, it is shown particularly that semen motility duration (SMD) plays a key role in terms of fertility soundness (Billard, 1978; Kozdrowski *et al*., 2007; Gennotte *et al*., 2012). The more gametes are motile, the more probability to reach the micropyle is important.

However, little is known concerning the variation of sperm motility duration throughout the reproduction season, and in our knowledge no reports have been published concerning existing relationships between SMD and CASA parameters. To date, SMD is still measured using optical microscopy with no automatic alternative. With CASA parameters being measured objectively, existing correlations with SMD could lead to potential fertility predictors in fish.

During the last decades, CASA systems have widely been developed generating a series of variables including the measurement of the percentage of moving spermatozoa, straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), beat cross frequency (BCF, Hz), linearity of a curvilinear path (LIN, %), straightness (STR, %), wobble (WOB, %) and amplitude of lateral head displacement (ALH, µm) (Tuset *et al*., 2008b; Tejerina *et al*., 2009; Gallego *et al*., 2013a). These systems are particularly useful in fish where the short duration of motility makes the assessment of sperm quality difficult by direct observation (Kime, 1999; Fauvel *et al*., 2010).

In different animal species, individual or a group of CASA parameters, including VCL (Larsen *et al*., 2000), VSL and ALH (Moore and Akhondi, 1996), VSL, VAP and VCL **(**Viveiros *et al*., 2010), and BCF (Billard and Cosson, 1992; Oliveira *et al*., 2013) are suggested as fertility predictors. In fish, only a few studies are dedicated to investigate relationships between CASA

parameters and fertility. However, as in other animal species, conclusions remain controversial with different parameters presented as fertility predictors, including VSL, VAP, VCL and BCF (Farrell *et al*., 1998; Larsen *et al*., 2000; Fernandez-Santos *et al*., 2011; Del Olmo *et al*., 2013; Oliveira *et al*., 2013). These controversial results are often related to the low number of females finally inseminated.

Based on the presented background, the current study was carried out to explore existing relationships between CASA parameters and motility sperm duration. The experimental protocol consisted of simultaneous semen motility analysis, during the reproduction season of *B. barbus callensis*, including the measurement of SMD and CASA motility parameters.

Materials and methods

Fish handling and gamete collection

The barbel (*B. barbus callensis*, Cyprinidae family) was collected from Agrioun River (Bejaia), located in Northeastern Algeria (36°36′54.25″ N and 05°22′04.33″ E). Sperm was collected during the spawning season, from June (corresponding to the full reproduction season) to August 2012 (the end of spawning period). The fish were captured using fishing rods connected to a transparent net with 2cm^2 mesh. Seventy (70) barbels were assessed during the study period (body weight ranged from 3.85 to 327.70 g and total length from 7.50 to 33.30 cm).

After drying and cleaning the genital papilla with a paper towel to prevent water contamination and initiation of sperm motility, a gentle abdominal pressure was applied to collect semen. The samples contaminated with faeces and urine were discarded (Gallego *et al*., 2013).

Semen motility analysis

Optical microscopy analysis

Sperm motility was evaluated visually for the percentage of motile spermatozoa after activation under optical microscopy at 10×40 magnifications. The total duration of semen motility was assessed using a timer immediately after activation; the sperm is considered as immotile when less than 5% sperm remain mobile (Tuset *et al*., 2008). Semen samples were simultaneously diluted at 1:1000 in fresh river water for motility activation and CASA analysis.

CASA motility analysis

The sperm freshwater mixture was analyzed using a Makler cell (Sefi-Medical Instruments, Israel). The CASA system consisted of a triocular optical phase contrast microscope (Nikon, Eclipse E200. Phase contrast 0.90 dry (Japan), equipped with a warming stage at 37°C and a Basler A312fs digital camera (Basler Vision Technologies, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analyzed using the Sperm Class Analyzer (SCA, 4.0, 2014) software

(Microptic S.L.; Barcelona, Spain). Sampling was conducted using x 10 negative phase contrast objective. Software settings were adjusted to fish sperm. The standard parameter settings were as follows: 25 frames/s; 05–90 μm^2 for head area; VCL>10 µm/s to classify a spermatozoon as motile.

The parameters assessed were straight line velocity (VSL), the average velocity measured in a straight line from the beginning to the end of track in µm/s, average pathway velocity (VAP), the average velocity of the smoothed cell path in µm/sec, the curvilinear velocity (VCL), the average velocity measured over the actual point-to-point track followed by the cell in µm/sec, the beat cross frequency (BCF), the frequency of the sperm head crossing the average path in either direction expressed in Hz, linearity (LIN, %), defined as the ratio VSL/VCL, straightness (STR, %), defined as the ratio VSL/VAP, which expresses the linearity of the average path, and ALH (µm), defined as the amount of lateral displacement of a sperm head along its spatial average trajectory.

Statistical analysis

Statistical analysis was performed using Stat view 5.0 software (Abaccus). All experiments were repeated at least three times. Values are expressed as mean ± standard error. Coefficients of correlation were used to explore the existing relationships between sperm motility duration (SMD) and CASA parameters. One-way ANOVA followed by equality of variance F test was used to compare values of each parameter. Differences were considered as statistically significant at $p<0.05$.

Results

Semen motility duration

Fig. 1 represents the variation of semen motility duration during the study period. A significant increase in semen motility duration was observed from 16 (64.44 ± 0.39 s) to 22 June (75.12 ± 0.38 s). A regular decrease was subsequently observed from 22 June to 15 July to reach a minimum value of 21.88 ± 0.37 seconds. From 15 July to 6 August, an enhancement of semen motility duration was observed to reach a value of 62 seconds. However, during the two last analyses only three males were collected. This period corresponded to the end of the spawning season.

CASA motility parameters

Fig. 2 represents the variations in CASA parameters during the study period. A perfect similarity was observed when comparing VSL (Fig. 2A) to motility duration (Fig. 1). The maximum and minimum values were reached simultaneously on 22 June and 15 July, with 18.51±0.92 and 11.50 ± 0.45µm/s for VSL and 75.12±0.38 and 21.88±0.37 seconds for semen motility duration. This relationship was expressed by the highest coefficient of correlation (r = 0.74).

Figs. 2B and 2C represent VAP and VCL variations, respectively.

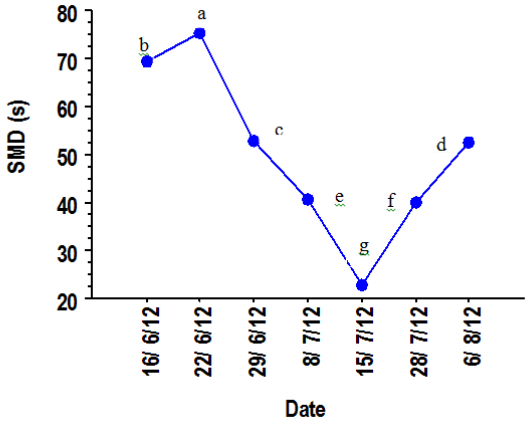

Figure 1: Semen motility duration (SMD) of barbel spermatozoa measured microscopically throughout the reproduction season. Values are expressed as means±SE. Different letters (a-g) indicate significant differences between groups on the basis of one-way ANOVA followed by equality of variance F test (p<0.05).

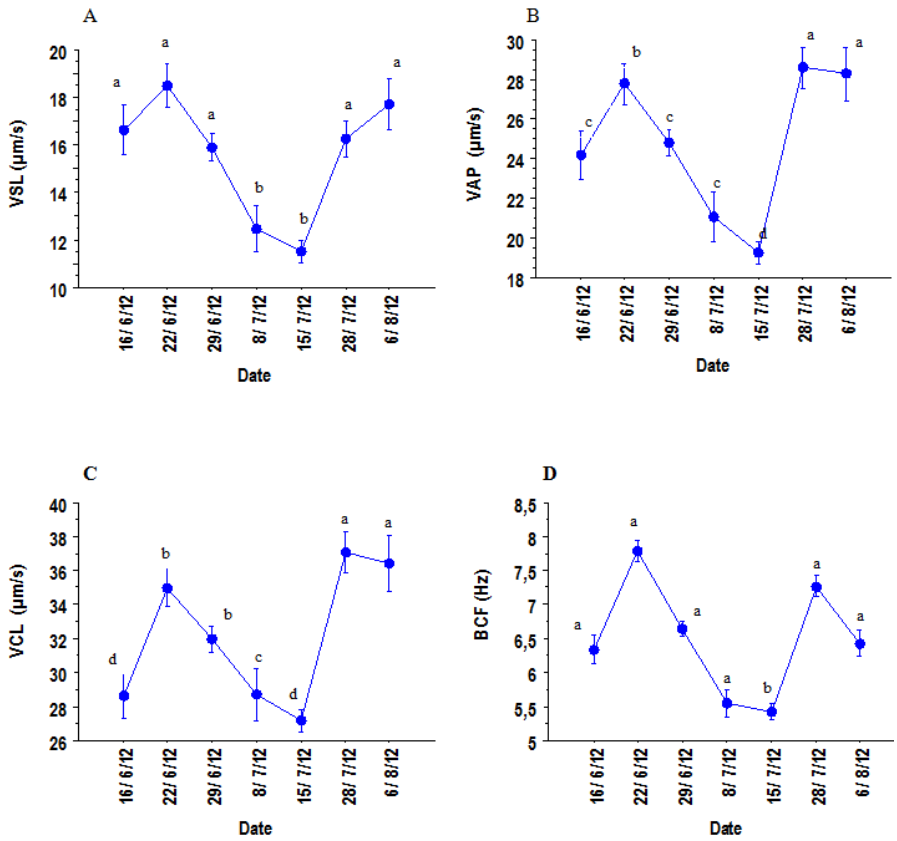

Figure 2: VSL (A), VAP (B), VCL (C) and BCF (D) values during the study period. Values are expressed as means±SE. Different letters (a-d) indicate significant differences between groups on the basis of one-way ANOVA followed by equality of variance F test (p<0.05).

VAP is a parameter calculated by smoothing VCL path. The basis of this measurement induced a similar evolution (r = 0.95) when comparing these two parameters. The minimum values for VAP and VCL were observed on 15 July at 19.26±0.54µm/s and 27.15±0.68µm/s, respectively. The maximum values were observed at the end of the reproduction season (28 July) at 28.61±1.03 µm/s and 37.11±1.23 µm/s, respectively. Coefficients of correlation with semen motility duration were 0.32 and 0.16, respectively. The beat cross frequency (BCF) (Fig.2D) showed a perfect similarity when compared to VAP and VCL with a simultaneous decrease between 28 July and 6 August. BCF was revealed, to some extent, to be positively correlated to SMD (r = 0.45). ALH, LIN and STR, showed no correlation with SMD (r = 0.006, r = 0.088 and r = 0.068, respectively, data not shown).

Discussion

Using computer systems, fish semen analysis is achieved readily generating numerous accurate parameters (Wilson-Leedy and Ingermann, 2007; Fauvel *et al*., 2010). However, it still remains controversial to predict fertility on the basis of CASA analysis (Amann and De Jarnette, 2012; Broekhuijse *et al*., 2012). In fish, semen motility duration, considered as a quantitative and objective parameter, is still generated using the conventional microscopic analysis. This parameter is revealed as a potent fertility indicator (Rurangwa *et al*., 1998; Kime *et al*., 2001). Nevertheless, to our knowledge, relationships with CASA motility parameters had never been explored. To investigate these relationships, semen was simultaneously analyzed microscopically and automatically by the aid of a CASA system during *B. callensis* spawning season. The main objective was to explore potential sperm quality and fertility indicators. During the study period, from 16 June to 6 August, semen motility duration showed an average value of 39.83 ± 25.24 seconds (Mean ± SD), values similar to those reported for cyprinids (Alavi *et al*., 2010) and sea bass (Abascal *et al*., 2007). Seven CASA parameters were measured: VSL, VAP, VCL, ALH, BCF, Linearity and Straightness. The mean recovered values, all samples considered, were 15.13±22.29µm/sec, 24.32±18.82 µm/sec, 31.8±22.29 µm/sec, 1.13 ± 0.79µm, 6.46 ± 3.26 Hertz, 44.86 ± 27.85 %, and 56.43 ± 28.05%, respectively. These CASA parameters, presented for the first time, could serve as reference expressing *B. barbus callensis* sperm motility during the reproduction season. As semen activation was achieved using fresh river water, such references remain of high importance, particularly in monitoring the impact of global environment changes on reproduction. CASA motility parameters differ vastly according to the analyzed species. The results showed that values for *B. barbus callensis* are lower than those reported for commune carp (*Cyprinus carpio*) but

remain similar to those reported for European eel (*Anguilla Anguilla*) with VSL = 12µm/s, VAP = 16 µm/s and VCL = 40 µm/s (Asturiano *et al*., 2004). Computer aided semen analysis has been used extensively in different domains in evaluating sperm quality in fish (Christ *et al*., 1996; Alavi and Cosson, 2005). However, it is still difficult to predict fertility outcome in an accurate manner (Amann and Dejarnette, 2012; Broekhuijse *et al*., 2012), mostly due to the deficiency of validated studies confronting CASA parameters and fertility outcomes. This remains difficult even in species such as human and bovine, where CASA is routinely used with continuous fertility feed-back after artificial insemination (Andersson *et al*., 1992; Barrat *et al*., 1993; Irvine *et al*., 1994; Januskauskas *et al*., 2000). Nevertheless, individual or a group of CASA parameters are reported as correlating significantly with fertility. Thus, it has been shown in rats that fertilizing capacity of spermatozoa correlates with straight line velocity (VSL) (Moore and Akhondi, 1996). In bulls (Farrell *et al*., 1998; Kathiravan *et al*., 2011; Oliveira *et al*., 2013) and humans (Barrat *et al*., 1993; Irvine *et al*., 1994; Krause, 1995; Larsen *et al*., 2000; Hirano *et al*., 2001), various parameters including VSL, VAP, VCL, BCF and ALH are reported as fertility predictors. In fish, only a few studies have been dedicated to investigate relationships between CASA parameters and fertility, in which contradictory results have been obtained. Thus, VSL, VAP, VCL and BCF are separately suggested to be correlated with fertility (Billard and Cosson, 1992; Lahnsteiner, 2000; Rurangwa *et al*., 2004; Viveiros *et al*., 2010). In fish, the identification of computer parameters that are strongly correlated with sperm quality and fertility is still pending. Alternative to fertility exploration, the present study was conducted to correlate CASA parameters with semen motility duration. When studying the evolution of semen motility duration throughout the reproduction season, no correlation had been observed with linearity, straightness and ALH (r = 0.06, 0.07 and 0.005, respectively). On the other hand, a perfect similarity was observed with VSL, VAP, VCL and BCF expressed by high coefficient of correlations (r = 0.74, 0.33, 0.16 and 0.44, respectively). The progressive movement, measured microscopically, is one of the most obvious parameters used in the assessment of sperm quality in fish **(**Kime *et al*., 2001). Its closer equivalent in CASA parameters is the straight line velocity (VSL). VSL corresponds to the average velocity measured by considering a straight line distance from the beginning to the end of spermatozoa track. It indicates the aptitude of the gametes to move forward in a straight line. This indicates that semen motility duration, expressing the aptitude of the gametes to be motile for a long period, expresses also the quality of the gametes motility to progress in a straight line, increasing thereby the probability to meet the oocytes.

Generally, the present study pointed out the interest of CASA motility parameters as potent indicators of sperm quality in fish. Significant correlations were established with semen motility duration, particularly concerning VSL. As VSL is reported previously in different animal species as a fertility indicator, this parameter could offer a real opportunity in fish. However, the present results need to be strengthened by further studies including simultaneous analysis of semen motility duration, CASA parameters and fertility outcomes.

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