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Spermatozoa Motility Parameters of the Pumpkinseed (*Lepomis gibbosus*) in a Standardized Solution and Ambient Water

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The sunfish are classified as an invasive species after their introduction into a new habitat. Milt quality and spermatozoa movement in several sunfish were described. The aim of this study was to determine the basic milt characteristics and spermatozoa motility parameters in pumpkinseed males after using ambient water and a standardized solution to examine the parameters in mimicked natural conditions and in controlled conditions allowing interspecies data comparisons. Nine spermatozoa motility parameters were traced using computer-assisted sperm analysis (CASA) after activation with 30 mM NaCl and ambient water at 20°C. Spermatozoa motility parameters on activation, except for ALH, differed after using saline solution and ambient water. The CASA parameters were higher in the first medium. On the other hand, spermatozoa motility was high in both media, with an average of 89.6 and 85.2% in saline solution and ambient water, respectively. In the media, initial spermatozoa motility was characterized by a mean curvilinear velocity of 122.4 and 108.5 μm s^{-1}, and a linearity of 79.7 and 67.1%, respectively. The active motility phase in the saline solution was approx. 8 min was longer than that measured in ambient water (mean 3 min). The pumpkinseed spermatozoa reveal good motility parameters, especially the high percentage of motile sperm, linearity, long duration of spermatozoa movement, as well as good velocity. It can be assumed that apart from reproductive strategy, spermatozoa motility parameters are valuable features of the male reproductive biology which may contribute to reproductive success.

Key words: *Lepomis gibbosus*, pumpkinseed, spermatozoa, motility, CASA.

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The pumpkinseed, *Lepomis gibbosus* (Actinopterygii: Perciformes: Centrarchidae), is a species that adapts well to different living conditions. It was introduced from North America and acclimated in many regions of other continents, spreading into adjacent water bodies (TOMEČEK *et al.* 2007). It has been reported as part of the ichthyofauna of at least 28 European countries and Asia Minor (COPP & FOX 2007). The invasiveness of the species is primarily related to its flexibility regarding habitat requirements and variety in life-history (ROBINSON & WILSON 1996; BERTSCHY & FOX 1999; TOMEČEK *et al.* 2007). Individuals of this species are found either in riverine or lacustrine environment, preferring shallow, weedy, quiet reservoirs, lakes, river inlets, but can also be found in river beds and side arms (MOLČIK 1962; HOLČIK 1995; ŠUMER *et al.* 2005; TOMEČEK *et al.* 2005). The pumpkinseed is a eurythermic species that can inhabit cool to moderately warm waters, preferring 20-30°C but tolerating temperatures as high as 36°C (ZAPATA & GRANADO-LORENCIO 1993; GUTIÉRREZ-ESTRADA *et al.* 2000; MISCHKE & MORRIS 2002). The species survives in waters with a pH range of 5.2-9.2 (SCHEUHAMMER & GRAHAM 1999; ŠUMER *et al.* 2005) and low dissolved oxygen concentration (FOX & KEAST 1990; KEAST & FOX 1990; OSENBERG *et al*. 1992; ŠUMER *et al.* 2005). Its survival in various ecosystems is also enabled by the plasticity in feeding mechanisms and flexibility in diet (ZAPATA & GRANADO-LORENCIO 1993; GARCÍA-BERTHOU & MORENO-AMICH 2000; BLANCO *et al*. 2003).

Another feature that contributed to the species' colonization success in new environments is its reproductive strategy. The pumpkinseed is characterized by a long reproductive season, continuous gamete production during the reproductive season, spawning in portions, and guarding the nest by the parental male (BURNS 1976; DANYLCHUK

& FOX 1994, 1996; VILA-GISPERT & MORENO-AMICH 1998, 2000; BERTSCHY & FOX 1999; COPP *et al.* 2002; NEFF *et al.* 2003; DOMAGAŁA *et al.* 2014). Moreover, early maturation of males is known in populations of *Lepomis*. The precociously mature males called cuckolders use sneaking or satellite tactics to fertilize eggs (GROSS 1979; STOLTZ & NEFF 2006a; NEFF & CLARE 2008; ALMEIDA *et al.* 2012). Only in *Lepomis macrochirus,* basic milt characteristics, spermatozoa motility parameters, ATP concentration in spermatozoa and investment in gonad development were studied considering differences in males using two alternative reproductive behavior patterns of parental males and cuckolders (LEACH & MONTGOMERIE 2000; NEFF *et al.* 2003; BURNESS *et al.* 2004, 2005; STOLTZ & NEFF 2006a, b). In some studies, differences in spermatozoa motility parameters between cuckolders and parental males were detected, while in other studies differences were not oberved (LEACH & MONTGOMERIE 2000; NEFF *et al.* 2003; BURNESS *et al.* 2005; STOLTZ & NEFF 2006b). There is no data on milt characteristics and spermatozoa motility parameters in other sunfish. The aim of this study was to determine the basic milt characteristics and spermatozoa motility parameters in pumpkinseed males after using ambient water and a standardized solution to examine the parameters in mimicked natural conditions and in controlled conditions allowing an interspecies data comparison.

Material and Methods

Animals and sperm collection

During the middle part of the spawning seasons in 2012, 2013 and 2014, on June 26, 24 and 16, respectively, up to 10-12 males of the pumpkinseed were caught each year in a canal with post-cooling water from the Dolna Odra power plant (NW Poland) (53°11'N, 14°29'E). In June, the maximum value of GSI in the population was noted (DOMAGAŁA *et al.* 2014). A total of thirty two males of the pumpkinseed were collected. The pumpkinseed were caught as bycatch by commercial fishermen and then transported in tanks to the laboratory of the University of Szczecin, where the milt was stripped. The volume of the milt was measured by aspiration into a calibrated syringe. No anaesthesia was used. The fish were managed by the fishermen and the experimental procedures used did not exceed the first degree of invasiveness (according to the Act on Experiments on Animals (Dz. U. 2005 No. 33 item 289). The fish were aged 2+ to 4+, and the age was determined based on the analysis of scale rings. Each year, a similar proportion of age classes was studied. The total length (LT) of the pumpkinseed individuals ranged from 9.2 to 16.2 cm (mean 13.05 cm, SD = 1.30) and the weight ranged from 11.2 to 74.2 g (mean 36.83 g, SD = 12.46).

Sperm motility analysis (CASA-computer assisted sperm analysis)

The analysis of spermatozoa motility parameters was performed in two activation media: (1) 30 mM NaCl, 20 mM Tris, pH 8.0, with the addition of 0.1% BSA (osmolality 80 mOsm kg^{-1}) to assess movement in a standardized solution, at 20°C; (2) ambient water from the canal where the fish live (osmolality 15 mOsm kg^{-1}, pH 8.0-8.2), to determine movement in natural conditions, at 20°C. Spermatozoa motility was evaluated on an automated system, Sperm Class Analyzer (SCA) v. 4.0.0. by Microptic S.L., Barcelona, Spain. Activation was triggered using a 100- to 200-fold dilution in the appropriate activating solution. The milt (1-2 µl) was added to 200 µl of the appropriate activating solution in a 1.5 ml polyethylene Eppendorf tube. The temperature of the solution in the tube was maintained using a cooling block (FINEPCR, Korea), at 20°C. After intense stirring of the tube contents for 2 to 3 s, 1.2 µl of such diluted solution was immediately placed into a well of a 12-well multitest glass slide (MP Biomedicals LLC, Eschwege, Germany) and covered with a coverslip. The microscope table was equipped with a cooling device (Semic Bioelektronika, Kraków, Poland) set at 20°C. The spermatozoa motility was monitored using a camera (Basler A312fc, with a CCD½" type sensor) recording at 50 frames per second, mounted on a Nikon Eclipse 50i negative phase contrast microscope linked with a CASA device, at 250× magnification. Half-second films (25 frames) were recorded at 10 s and every 30 s until 160 s of swimming, and then at 5, 7 and 12 min until active swimming ceased. The activation and film recording were repeated three times per individual. Two milt samples of less than 5 µl were excluded from the analysis due to the inability to perform repetitions of the CASA analysis. Nine parameters characterising motility were chosen for analysis: 1. MOT – percentage of motile spermatozoa (with an average path velocity >20 µm s^{-1} as the criterion of motility); 2. VCL – curvilinear velocity (µm s^{-1}); 3. VAP – average path velocity (µm s^{-1}); 4. VSL – straightline velocity (µm s^{-1}); 5. LIN – linearity (VSL/VCL×100); 6. STR – straightness (VSL/VAP×100); 7. ALH – amplitude of lateral head displacement (µm), i.e. magnitude of the lateral movement of the spermatozoan head with respect to its average track; 8. BCF – beat cross frequency (Hz), i.e. the track crossing frequencies or the average frequency at which a sperm cell's curvilinear track crossed its average track; and 9. motility duration – time from activation to cessation of active movement by the spermatozoa.

The spermatozoa concentration was assessed on a CASA system using a Bürker chamber and a dilution of 2000× in 0.8% NaCl (samples with a volume of less than 10 µl were discarded from the analysis).

Statistical Analysis

The data were checked for normality using the Shapiro-Wilk test**.** A nonparametric Wilcoxon test was used to determine the significance of the differences in motility parameters between saline solution and ambient water activation. The nonparametric Wilcoxon test was used to find the significance of the differences in motility parameters in the saline solution and ambient

water The nonparametric multiple comparison ANOVA Kruskal-Wallis test was used to assess the significance of the differences in the motility parameters at different intervals in the medium. The Spearman correlation was used to check the statistical significance of the correlations observed. All statistical procedures were performed with the Statistica 10.0 software. The results were regarded as statistically significant at a level of 0.05.

Results

The percentage of motile sperm differed significantly between the activating media. On activation, MOT was higher in 30 mM NaCl (mean 89.6±9.8%, range 62-100%, n=30) compared to ambient water (mean 85.2±9.9%, range 60-96%, n=30) (Fig. 1a). In the saline solution, the percentage of motile sperm decreased more slowly than in water, therefore the average duration of the active spermatozoa motility phase in the solution was longer than that in the water, 474.0±148.7 s and 186.3±91.5 s, respectively (P<0.001, Wilcoxon test). In the first phase of movement, the velocities VCL, VAP and VSL were significantly higher after activation with 30 mM NaCl than those in water. At 10 s post-activation, the mean velocities in the saline solution reached 122.4±18.3, 111.9±21.4 and 98.6±24.1 µm s^{-1}, respectively, and those in water reached 108.5±19.1, 94.9±22.9 and 73.4±17.2 µm s^{-1}, respectively (Fig. 1b, c, d). During the first minute of the motile phase, the velocities VCL and VAP decreased rapidly, and from 100-160 s of movement phase, the parameters became similar in the two media. In the first phase of movement, the linearity (LIN) and straightness (STR) of spermatozoa movement were higher in the saline solution than in water and gradually decreased in the motile phase. On activation, LIN was of 79.7±13.5 % and 67.1±8.9% in the saline solution and water, respectively, while STR was as high as 86.7±9.4 and 76.6±7.3%, respectively (Fig. 1e, f). The amplitude of lateral head displacement (ALH) slightly fluctuated over the motility phase (1.18-1.42 µm), being similar on activation and in the second phase of movement in the two media (Fig. 1g). In the first phase of movement, beat cross frequency (BCF) differed in both media, being higher in the saline solution, while in the second half of movement, the parameter was similar in the two media. In the saline solution, BCF slightly increased from 17.6 Hz at t=10 s to 21.9 Hz at t=70 s and then decreased, while in water the value ranged from 11.2 to 18.1 Hz (Fig. 1h).

The average amount of milt that occurred in the reproductive tract of the pumpkinseed individuals was 92.1±154.8 µl, but the value ranged from 1 to 600 µl (n=32). The spermatozoa concentration in the investigated individuals also showed high variation, from 3.3 to 23.8 × 10^9/ml, with a mean of 10.0 ± 5.7 × 10^9/ml (n=25). In the collected samples, significant positive correlations between fish length and milt volume (rs=0.42, P<0.05, Spearman correlation), and between fish length and duration of spermatozoa movement (rs=0.48, P<0.05, Spearman correlation) were found. A negative correlation between fish length and spermatozoa concentration was detected (rs=-0.48, P<0.05). No correlation was found between fish length and curvilinear velocity (rs=0.28, P>0.05), and MOT (rs=0.02, P>0.05) and other CASA parameters measured.

Discussion

It is known that the motility parameters measured in CASA are affected by the composition of the activation solution (MARTÍNEZ-PASTOR *et al.* 2008; DZIEWULSKA & DOMAGAŁA 2013). The main factors influencing the nature of spermatozoa movement occurring in the solutions are osmolality, pH, CO_2 content, ion composition organic content and temperature (LAHNSTEINER 2002; ALAVI & COSSON 2005, 2006; COSSON *et al.* 2008a). Another factor affecting the parameters measured in CASA is the technique of sample handling (CHAPIN *et al.* 1992; DZIEWULSKA *et al.* 2011; BORYSHPOLETS *et al.* 2013). In the presented study, spermatozoa activation was conducted under controlled conditions, allowing comparison of the results with other studies, and in conditions mimicking the natural environment to evaluate motility in the ambient water environment. On activation, the values of the spermatozoa motility parameters obtained in 30 mM NaCl were higher than those in ambient water (except for ALH). The differences in the CASA parameters measured in the pumpkinseed in these two media

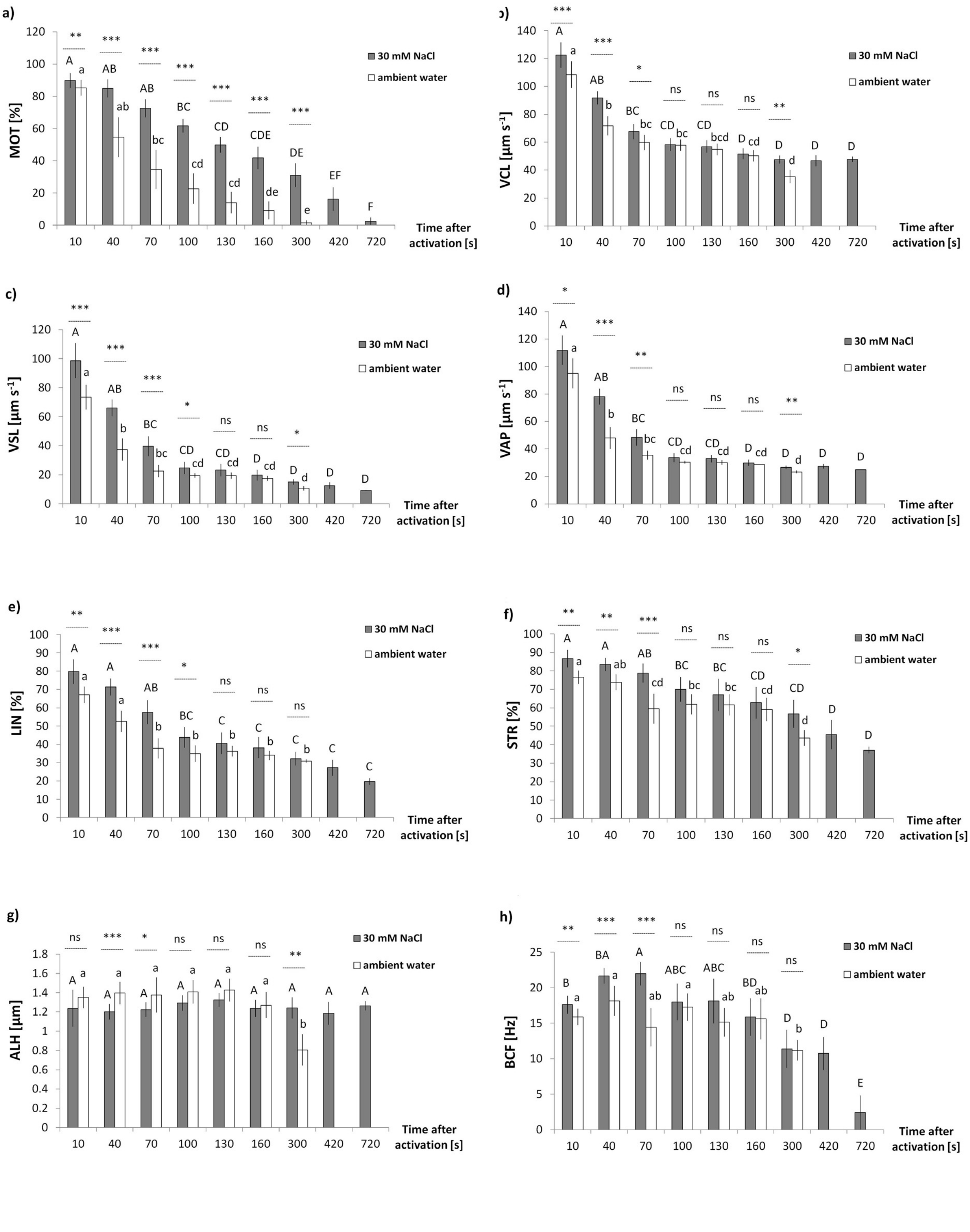

Fig. 1. Motility parameters of the pumpkinseed spermatozoa activated with 30 mM NaCl and ambient water at 20°C at particular times after activation (a) MOT – percentage of motility, (b) VCL – curvilinear velocity, (c) VAP – average path velocity, (d) VSL – straight line velocity, (e) LIN – linearity, (f) STR – straightness, (g) ALH – amplitude of lateral head displacement, (h) BFC track crossing frequencies (n=30). Mean value ± SEM. A nonparametric Wilcoxon test was used to find the significance of the differences in motility parameters in the saline solution and ambient water. Asterisks indicate the level of statistical significance (*$P<0.05$; **$P<0.01$; ***$P<0.001$). The nonparametric multiple comparison ANOVA Kruskal-Wallis test was used to assess significance of differences in motility parameters in different intervals of time after activation in saline solution (A,B,C –– values marked with different letters are significantly different, $P<0.05$) and ambient water (a,b,c – values marked with different letters are significantly different, $P<0.05$).

were similar to those found in the whitefish (DZIEWULSKA *et al.* 2015a). The lower percentage of motile spermatozoa and lower speed of the gametes noted in water could be caused by the adhesion of sperm to the glass surface because the medium was not supplemented with protein. This effect was shown in carp spermatozoa activation in media of different protein supplementation (KOWALSKI *et al*. 2014). On the other hand, despite the differences in MOT in the media, spermatozoa motility in the studied pumpkinseed was high in both solutions. In most males, the spermatozoa motility exceeded 80%. Mean MOT of 85.2% in water was higher than that noted in *L. macrochirus*, 70-80% in lake water after two step dilution (BURNESS *et al.* 2004, 2005). In ambient water, the motility of spermatozoa of the studied *L. gibbosus* lasted approximately 3 min, and in the saline solution, approx. 8 min. The shorter swimming time and the faster decrease of MOT in water, compared to that in the prepared solution, are typical of spermatozoa movement in media of lower osmolality (BILLARD 1978; DIETRICH *et al.* 2007; ALAVI *et al.* 2006, 2009; ŻARSKI *et al.* 2012; DZIEWULSKA *et al.* 2015a). The duration of motility in *L. gibbosus* was slightly longer than in *L. macrochirus,* reaching approx. 2 min in lake water at 20°C (BURNESS *et al.* 2005). *Lepomis* is characterized by a longer motility phase compared to most other freshwater fish in which the movement usually does not exceed 2 min (COSSON *et al.* 1991; MANSOUR *et al.* 2003; LAHNSTEINER *et al.* 2004). The duration of spermatozoa movement in fish inhabiting the Oder River measured in the same controlled conditions at a temperature specific for the species reproductive season does not exceed 3 min, even though the spawning temperature for native species was lower (DZIEWULSKA *et al*. 2015 a, b; authors' unpublished data). The motility phase is prolonged only in Acipenseridae (TOTH *et al.* 1997; COSSON *et al.* 2000). In marine fish, the duration of motility is extended to a few minutes, but the spermatozoa of these species are introduced into a hyperosmotic environment in which no water absorption is observed (CHAUVAUD *et al.* 1995; COSSON *et al.* 2008b). In fish with long-moving spermatozoa, ATP concentration remains at a stable level of 30% of the initial value in the second phase of movement (BILLARD *et al.* 1999; DREANNO *et al.* 1999; BURNESS *et al.* 2005).

The velocity obtained by the spermatozoa of *L. gibbosus* was similar to that reported for *L. macrochirus* at 20°C (BURNESS *et al.* 2005) but lower than that obtained in a previous study by the same group (BURNESS *et al.* 2004). BURNESS *et al.* (2005) observed an increase in spermatozoa motility and velocity up to 40 s of the duration of the motility phase post-activation. In the pumpkinseed individuals investigated in this study, both parameters were the highest in the first detected image. It is difficult to compare the spermatozoa velocity of fish spawning at different temperatures since the velocity value is more than doubled when the temperature increases by 10°C (COSSON *et al.* 2008a). The velocity achieved by spermatozoa in water environment affects the time at which male gametes reach the egg. The attained velocity of spermatozoa in *L. gibbosus*, compared to fish spawning in the Oder River at a lower temperature, is close to the velocity of Percidae and Salmonidae and is higher than in Cyprinidae, measured in the same controlled conditions at a temperature specific for the species reproductive season (DZIEWULSKA *et al*. 2015 a, b; authors' unpublished data). The spermatozoa of *L. gibbosus* start moving with good straightness, better than or similar to that of other fish (BURNESS *et al.* 2004; DZIEWULSKA *et al.* 2015a, b; authors' unpublished data). In the studied pumpkinseed, the values of LIN and STR gradually decrease as the duration of movement increases, while the gametes in *L. macrochirus* swim with increasing straightness throughout their active period (BURNESS *et al.* 2004).

In *Lepomis*, as in other fish with early maturation in males, alternative reproductive tactics are known (GROSS 1979; UGLEM *et al.* 2001; VLADIĆ & JÄRVI 2001; NEFF & CLARE 2008). Smaller males (cuckolders) employ sneaking or satellite tactics to compete with the parental male to fertilize eggs. In sunfish, the parental male builds a nest and provides care for the offspring in the nest (GROSS 1979; STOLTZ & NEFF 2006a; NEFF & CLARE 2008). Parental males had the largest testes but the smallest relative to their body weight, but also longer-lived sperm and lower sperm concentration in the ejaculate compared to younger males (LEACH & MONTGOMERIE 2000; NEFF *et al.* 2003; BURNESS *et al.* 2005). Higher ATP concentration in precocious males compared to parental males was also reported (BURNESS *et al.* 2004, 2005). The percentage of motile sperm and straightness did not differ in males with alternative reproductive tactics (BURNESS *et al.* 2004, 2005). In some studies, spermatozoa velocities were noted to be higher in younger males (BURNESS *et al.* 2004), but in further studies, no differences were seen (BURNESS *et al*., 2005). STOLTZ and NEFF (2006b) did not find any difference in the curvilinear swim speed, path linearity or sperm flagellum length among the three male mating types. In different environments, the sunfish exhibit a high degree of variation in size and age at maturation (ALMEIDA *et al.* 2012). In the studied population of the pumpkinseed, investigation of the male reproductive behavior has not been conducted yet. In our sample, a positive correlation was found between fish length, milt volume and duration of spermatozoa movement, while a negative correlation was detected between fish length and spermatozoa concentration. The obtained correlations reflect similar trends of basic milt characteristics in relation to fish size associated with alternative reproductive tactics discussed above.

The pumpkinseed spermatozoa reveal good motility parameters, especially the high percentage of motile sperm, linearity, the long duration of spermatozoa movement as well as good velocity. Two CASA parameters, the percentage of motile spermatozoa and spermatozoa velocity, have been found to be positively correlated with fertilization efficiency in other species (BILLARD *et al.* 1986; LAHNSTEINER *et al.* 1998; GAGE *et al.* 2004; RURANGWA *et al.* 2004) and can be a determinant of the quality of gametes. It can be assumed that apart from the reproductive strategy of the pumpkinseed, the spermatozoa motility parameters are valuable features of reproductive biology which may contribute to reproductive success.

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