https://doi.org/10.52973/rcfcv-e35636 Revista Científica, FCV-LUZ / Vol. XXXV

Assessment of semen quality in freshwater mussel *Unio elongatulus eucirrus*: analysis of sperm motility, morphometry, and pH

Evaluación de la calidad seminal en el mejillón de agua dulce *Unio elongatulus eucirrus*: análisis de la motilidad, morfometría espermática y pH

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ABSTRACT

The freshwater mussel (*Unio elongatulus eucirrus*), native to the Euphrates River basin in Türkiye, holds little economic value; however, it plays an important ecological role in natural aquatic ecosystems. Consequently, understanding its biology particularly its reproductive traits, such as semen quality, sperm motility, and morphology is of scientific interest. This study aimed to investigate several reproductive parameters in 10 male specimens of this species, including sperm concentration, seminal pH, motility characteristics, and sperm morphometry. Sperm kinematics were assessed using a computer-assisted sperm analysis (CASA) system. The results showed the following sperm velocity values: straight-line velocity (VSL: 40.75 ± 6.01 µm/s), curvilinear velocity (VCL: 103.00 ± 2.62 µm/s), and average path velocity (VAP: 54.24 ± 6.75 µm/s). Sperm morphometry, analyzed via scanning electron microscopy (SEM), revealed a head length of 3.90 ± 0.11 µm, head width of 1.70 ± 0.17 µm, and tail length of 37.64 ± 0.45 µm. Regarding the physicochemical parameters, the seminal pH was 6.25 ± 0.26, and sperm concentration was 15.48 ± 0.53 × 10^9 cells/mL. These results indicate that the morphological and kinematic characteristics of freshwater mussel (*Unio elongatulus eucirrus*) spermatozoa are quite like those observed in other mussel and fish species. However, it was determined that spermatozoa are easily activated upon contact with water.

Key words: Freshwater mussel; sperm cell characteristics; Euphrates River Basin; Türkiye

RESUMEN

El mejillón de agua dulce (*Unio elongatulus eucirrus*), que habita en la cuenca del río Éufrates en Turquía, carece de importancia económica; no obstante, representa un valioso aporte ecológico en los ecosistemas acuáticos naturales. Por esta razón, resulta de interés conocer su comportamiento, especialmente en lo relacionado con la reproducción, lo cual incluye la calidad seminal, en particular la motilidad y la morfología espermática. El objetivo de este estudio fue investigar algunos parámetros reproductivos en 10 individuos machos de esta especie, tales como la concentración espermática, el pH seminal, la cinemática y la morfometría de los espermatozoides. La cinemática espermática fue determinada mediante un sistema de análisis asistido por computadora (CASA) bajo microscopio. Los resultados obtenidos revelaron los siguientes valores: velocidad rectilínea (VSL: 40,75 ± 6,01 µm/s), velocidad curvilínea (VCL: 103,00 ± 2,62 µm/s) y velocidad media de trayectoria (VAP: 54,24 ± 6,75 µm/s). La morfometría fue evaluada mediante microscopía electrónica de barrido (SEM), obteniéndose las siguientes dimensiones: longitud de la cabeza (3,90 ± 0,11 µm), ancho de la cabeza (1,70 ± 0,17 µm) y longitud de la cola (37,64 ± 0,45 µm). En cuanto a los parámetros fisicoquímicos, el pH seminal fue de 6,25 ± 0,26, mientras que la concentración espermática alcanzó los 15,48 ± 0,53 x 10^6 células/mL. Estos resultados indican que las características morfológicas y cinemáticas de los espermatozoides del mejillón de agua dulce (*Unio elongatulus eucirrus*) son bastante similares a las observadas en otras especies de mejillones y peces. No obstante, se determinó que los espermatozoides se activan fácilmente al entrar en contacto con el agua.

Palabras clave: Mejillón de agua dulce; características de los espermatozoides; cuenca del río Éufrates; Turquía

Recibido: 08/02/2025 Aceptado:15/04/2025 Publicado: 13/05/2025

INTRODUCTION

The mussels have contributed to aquatic ecosystem include that are biofiltration, enviromental monitoring, habitat modification, nutrient cycling and food webs [1]. In addition, they are important components of the aquatic ecosystem because they constitute a large part of the benthic biomass, play major functions in water filtration, water clarity, play a role in biogeochemical cycles and sedimentation, and support biodiversity [2]. Despite their important role in the ecosystem, mussels are rapidly declining worldwide, and 224 of the 511 freshwater mussel species (nearly half) are classified as Near Threatened on the 2015 IUCN Red List of Threatened Species [3]. Otherhand, Çetinkaya [4] reported that the freshwater mussels have lived totally 5 species such as Unio spp (2 species)., *Sinanodonta* sp., *Legumina* sp. and *Pseudodontopsis* sp. in Euphrates River in Türkiye.

The low reproductive capacity is generally a limiting factor on reproductive efficiency of aquatic organisms in their environment [5]. There is a positive correlation between the motility and velocity of sperm cells. Therefore, the decreasing in these parameters may reduce the reproductive efficiency [6]. Other hand, the motility and sperm cell motility descriptors which are indicator for sperm quality are determined by the computer assisted sperm analysis (CASA). Because, this instrument is the most objective according to traditional investigation methods and facilitates rapid assessment [7]. Even, generally, the motility of sperm cells can live only few minutes, even less than 1 min after contaminate with water [8]. Due to the short duration of motility, it is crucial that operation and study be rapidly accomplished. It is why more investigators started to use CASA systems and its usage has been growing up all over the world [9].

Some spermatological characteristics have been described on bivalves such as *Ruditapes philippinarum*, *Crassostrea gigas* and *Patinopecten yessoensis* [10], *Truncilla truncate* [11], *Neotrigonia margaritacea* [12], zebra mussel, *Dreissena polymorpha* [13 ,14]. But there is not any study about sperm cell motility descriptors and morphology of sperm cell in *Unio elongatulus eucirrus* in literature. The taxonomy of this species is that Phylum: Mollusca, Class: Bivalvia, Family: Unionidae, Genus: *Unio,* Species: *elongatulus,* Subspecies: *eucirrus* [15]. Thus, this research aimed to determine the external morphological characteristic and sperm motility descriptors of freshwater mussel species, *Unio elongatulus eucirrus.*

MATERIAL AND METHODS

Animal collection and husbandry for sperm samples

For sperm samples at the end of April 2024, the mature males of *Unio elongatulus eucirrus* were collected on the shores of Karakaya Dam Lake (38°29’26.0”N 38°21’23.7”E) on Upper Part of the Euphrates River in Malatya, Türkiye. The mussels kept in an aquarium (60 L) with tap water non-stop aerated (23 °C same like water temperature of lake). The body of each mussel was wiped dry to avoid any contamination with water before sperm samples collection. Sperm samples were collected into Eppendorf tubes from 10 individuals’ mussel and kept on ice for the duration of the experiment [14].

The kinematics and concentration of sperm cells

The kinematics of sperm cells in such as VSL: straight line velocity (µm/s), VCL: curvilinear velocity (µm/s), VAP: angular path velocity (µm/s), LIN, linearity (%), the ratio of net distance moved to total path distance, BCF: beat cross frequency turning points of the sperm cell head (Hz), ALH: amplitude of lateral displacement of the sperm cell head (µm) and MAD: mean angular displacement (°), average change in direction of the sperm head from frame to frame [8 , 16] were carried out by the computer assisted sperm analysis systems, BASA-Sperm Aqua which has Olympus CX31 microscope (Japan) with 200x magnification lens, Sony CCD camera (Japan) with 30 frames/second. The sperm samples from 10 males were activated with activation solution (AS) at a ratio 1:250 (Sperm: AS). Activation medium was aquarium water (23 ^{0}C, pH 7.9). Sperm cell concentration (sperm cell/mL) was determined from males by using a Neubauer hemocytometer (Germany) [17]. The sperm pH in testis was determined pH indicator papers (Merck Millipore, Germany).

The morphology of sperm cells

For scanning electron microscope (SEM) imaging, sperm samples were collected by pipette, concentrated by centrifugation (Hettich Universal 320R Refrigerated Centrifuge, Germany) and fixed for 3 h in freshly prepared 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 0–4 °C. Then the samples rinsed in the same buffer and immersed for 2 h in 1.0% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 20 °C. After rinsing in the buffer, the samples were transferred critical point drying apparatus. The dried samples were coated with gold palladium and imaged using a LEO EVO40 model scanning electron microscope (Oberkochen, Germany) at accelerating voltages of 20 kV [18]. The length and width of head and tail length were determined on 25 spermatozoa specimens from scanning electron micrographs. The photographs of sperm cells were also taken from 100 x lenses, immersion oil of light microscopy by staining Diff-Quick. In staining, the samples immersed in Diff-Quick A solution for 10 sec, Diff-Quick I solution for 5 seconds and Diff-Quick II solution for 5 sec. Then slides were washed with water, dried air for 1 min. It is described spermatozoa dimensions from photographs by Corel Draw software.

Statistics analysis

The SPSS 17 software was used for statistical analysis. All parameters were expressed as mean ± standard deviation (Mean±SD) and the statistically significant level was accepted as $P < 0.05$. The homogeneity of the data in the parameters was tested using Skewness and Kurtosis in descriptive analysis.

RESULTS AND DISCUSSION

The kinematics, concentration, head length, head width and tail length of sperm cells and pH of sperm in *Unio elongatulus eucirrus* have shown in TABLE I. The pH of the sperm in the testicle is slightly acidic.

TABLE I. Sperm parameters of Unio elongatulus eucirrus

Sperm parameters (n=10)	Mean ± S.D.	Minimum	Maximum
Concentration of sperm cell (x10^9/mL)	15.48±0.53	14.80	16.10
pH of sperm	6.25±0.26	6.00	6.5
VSL (µm/s)	40.75±6.01	31.39	51.67
VCL (µm/s)	103.00±2.62	99.54	118.63
VAP (µm/s)	54.24±6.75	49.48	68.26
LIN (%)	30.22±6.30	22.20	44.73
BCF (Hz)	5.92±1.52	3.27	8.76
ALH (µm)	23.97±7.94	11.49	33.75
MAD (°)	0.04±0.02	0.01	0.09
Head length of sperm cell (µm)	3.90±0.11	3.66	3.99
Head width of sperm cell (µm)	1.70±0.17	1.35	1.92
Tail length of sperm cell (µm)	37.64±0.45	36.95	38.44

VSL, straight line velocity (µm/s), VCL, curvilinear velocity (µm/s), VAP, angular path velocity (µm/s), LIN, linearity (%), BCF, beat cross frequency (cross/second), ALH, amplitude of lateral displacement of the spermatozoa head (µm), MAD: mean angular displacement (°), average change in direction of the sperm head from frame to frame.

The morphological characterization of sperm cell in *Unio elongatulus eucirrus* has shown in FIG. 1. The head of sperm cells has formed to approximately elongate cylindrical or bulled in the shape which has length 3.90±0.11 µm and width 1.70 ± 0.17 µm. The flagellum (lenght 37.64±0.45 µm) was very long as 10 times longer than head (TABLE I).

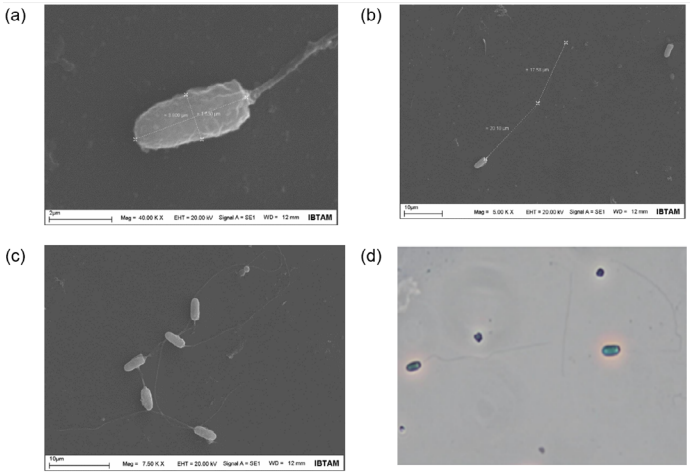

FIGURE 1. Figure a, b and c show images of different magnifications in scanning electron microscope (SEM) micrograph. Figure d shows image in 100x lens of light microscopy by staining Diff-Quick in freshwater mussel, Unio elongatulus eucirrus sperm cells

The structure of both morphology and ultra-structure of sperm cell in fish has been used to establish phylogenetic relationships among species [19 , 20]. However, the identified character of head morphology in sperm cells can be used to both predict semen quality and an indicator for fertility [21]. Assessment of sperm cell head morphology is also important in aquaculture for cryopreservation and motility [22 , 23].

Spermatozoa are immotile in the testis, however, the activation of sperm motility in the freshwater mussel, *Unio elongatulus eucirrus* is the same as fish and other aquatic invertebrates (Video S1). According to the obtained parameters, sperm cells which generally move on a straight or slightly curved route after activation which found to be similar to and / or closely related to the results of some authors [6 , 10 , 24 , 25].

The formation and structure of mussel sperm cell have been studied mostly in marine bivalves, but have been less in particularly freshwater bivalves. Some of the scientific studies on sperm characteristics of freshwater mussels are as follows. For example, it was studied about concentration and morphology of sperm cell two freshwater unionid mussel such as *Ligumia subrostrata* and *Ligumia straminea*. Researchers focused especially differences in seasonal. According to their results, largest sperm cell concentration of *L. subrostrata* was 19.60 × 10^9 cells/mL in between end of September to start of November. Greatest sperm concentration of *L. straminea* was 20.0 × 10^9 cells/mL on September. They reported that the sperm cells for both species were uniflagellated. Other hand, the dimensions of sperm cell in scanning electron microscopy (SEM), the mean head length and width (mid-spawning) of sperm cell were 3.38 ± 0.04 µm for *L. subrostrata* and 1.61 ± 0.01 µm and 3.37 ± 0.04 µm and 1.61 ± 0.01 µm for *L. straminea* [17]. At other study, it was researched about reproductive cycle, fecundity and growth of the freshwater mussel *Unio tumidus* (Bivalvia: Unionidae). Researchers reported the five main stages in spermatogenesis such as spermatogonia, spermatocytes, spermatids, spermatozoa and spermatid morulae. They determined the spherical shaped cell in all stages of spermatogenesis. The diameter of spermatogonia was 3.6–4.8 µm, while the head diameter of spermatozoa was 2.1 µm and bullet-shaped [26]. Moreover, the sperm dimensions of some freshwater unionid mussels were the head of spermatozoa measured 1.5 × 4.0 µm cylindrical and the 35 µm long flagella for *Anodonta grandis* [27], 4.0 µm long and 1.5 µm wide with 20-24 µm long flagella for *Diplodon chiliensis chiliensis* [28]. The dimensions of head and tail of spermatozoa and the cell density obtained in this study are parallel to sperm dimensions of those freshwater mussels presented in the literatures [17 , 26 , 27 , 28].

Other hand, the researchers' studies about spermatozoa morphology about four marine bivalve species from the Beibu Gulf of the South China Sea. According to their results these

bivalves had cone-shaped of sperm. The length-width of head and the length of tail in sperm of these bivalves were found for *Paphia schnelliana* (2.26-1.59 µm and 47.37 µm), *Lutraria sieboldii* (1.76-1.55 µm and 35.38 µm), *Antigona lamellaris* (2.84-1.26 µm and 38.19 µm), and *Paphia textzle* (2.59-1.09 µm and 45.95 µm) [29]. A deep-sea bivalve, *Calyptogena pacifica* has bullet-shaped sperm head and measured about 5.8 µm in length and 1.8 µm in width of sperm head [30]. The dimensions of spermatozoa in *Arctica islandica,* a marine bivalve from the North Atlantic had the rod-shaped 'head' regions (= acrosomal complex plus nucleus) about 9-9.5 µm on head, about 60 µm of flagellum and tapered, rod-shaped, while *Neotrapezium sublaevigatum*, a marine bivalve from Indo-Pasific from had 9.5 µm of spermatozoa head and short, barrel shaped [31]. In the present study, there was differences in total sperm head width and length, flagella length and spermatozoa shape amongst other marine bivalves. The gamete quality and the dimensions or morphology of spermatozoa can be highly variable among invertebrates. This situation has also been among populations of the same species [29 , 30].

CONCLUSIONS

Finally, it has been documented the presence of sperm characteristics of *Unio elongatulus eucirrus* in this manuscript. Overall, the results from the present study indicate there is compatible or similar with the semen characteristics and the dimensions of spermatozoa in some freshwater mussels living in other habitats. Additionally, in the present study, there was development of a rapid and reliable technique to quantify sperm motility using computer assessment sperm analysis system.

We have also hoped that this data may lead to understand to reproduction physiology of this species. Further studies are necessary to clarify the exact time, ions, temperatures and fertilization capacities of sperm activation in this freshwater mussel species.

ACKNOWLEDGEMENTS

The Department of Fishery Engineering, Faculty of Agriculture in Malatya Turgut Özal University for providing laboratory equipment of the work described in this paper.

Funding

No funding was received for conducting this study.

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