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A Novel Computer Assisted Sperm Analyzer for Assessment of Spermatozoa Motility in Fish; BASA-Sperm Aqua

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Abstract: This study was conducted to determine the working principle and operability of the BASA-Sperm Aqua module software of the newly developed computer-assisted sperm analysis system (BASA) for the evaluation of spermatozoa motility in fish. Semen samples of trout (*Oncorhynchus mykiss*) species were examined for this purpose. Sperm motility parameters such as VSL (µm/s), VCL (µm/s), VAP (µm/s), LIN (%), BCF (Hz), ALH (µm) and MAD (°) were examined. The investigated parameters were compared with data which analyzed in similar computer systems and published in international manuscripts. Finally, the BASA-Sperm Aqua has been found to be a software that performs its functions very quickly and practical and produces accurate and understandable results in the determining sperm quality parameters of fish.

Keywords : BASA-Sperm Aqua, computer assisted sperm analyzer, rainbow trout

Balıklarda Spermatozoa Motilite Değerlendirmesi için Yeni Bir Bilgisayar Destekli Sperm Analizörü; BASA-Sperm Akua

Öz : Bu çalışma, yeni geliştirilen bilgisayar destekli sperm analiz sisteminin (BASA Sperm), balıklarda spermatozoa motilitesinin değerlendirilmesi için oluşturulan BASA-Sperm Aqua modül yazılımının çalışma prensibini ve çalışabilirliğini belirlemek amacıyla yapılmıştır. Bu amaçla gökkuşağı alabalığına (*Oncorhynchus mykiss*) ait semenörnekleri incelendi. Spermatozoa motilite değerlendirmesi içerisinde VSL (µm / s), VCL (µm / s), VAP (µm / s), LIN (%), BCF (Hz), ALH (µm) ve MAD (°) gibi parametreler incelenmiştir. İncelenen parametreler, benzer bilgisayar sistemlerinde analiz edilen ve uluslararası makalelerde yayınlanan verilerle karşılaştırılarak değerlendirildi. Sonuç olarak, BASA Sperm Akua yazılımı, fonksiyonlarını çok hızlı ve pratik bir şekilde yerine getiren ve balıkların sperm kalitesi parametrelerinde doğru ve anlaşılır sonuçlar üreten bir yazılım olduğu görülmüştür.

Anahtar kelimeler: BASA-Sperm Aqua, bilgisayar destekli sperm analizörü, gökkuşağı alabalığı

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1. Introduction

1.1. Why is semen analysis necessary?

The determining quality of semen is necessary to identify its quality. However, quality determination always requires practical methods. Many parameters such as percentage of active spermatozoa, motility duration, spermatozoa concentration, spermatocrit and seminal plasma composition are used to measure semen quality [1]. The new larvae is desired to have high ability to live, resistant to diseases, well-nourished and fast growing that they can only be achieved by using breeders with high gamete quality (sperm and eggs) in fish production and aquaculture [2,3]. However, in commercial fish hatcheries, semen is often inadequate in both quality and quantity and it does often not have sufficient fertilization ability during artificial insemination. For example, African catfish (*Clarias gariepinus*), turbot (*Psetta maxima*) and yellowtail flounder (*Pleuronectes ferrugineus*) have not enough sperm volume (Less than 1 ml / 1 fish) [4,5].

Therefore, the determining quality of semen is very important and it is necessary to know its quality. But we need to get practical methods to determine sperm quality parameters such as velocities, movement styles, concentration, duration, motility duration.

1.2. Which methods are available for semen analysis?

The assessment of fish sperm motility was assessed over time by subjectively estimating motility characteristics expressed as percent of motile sperm cells and total motility duration, or a combination of both [6,7]. The percentage of motile spermatozoa and swimming power generally give an arbitrary score equivalent from 0 (immotile) to 5 (the whole spermatozoa is strongly moving) [6]. Less subjective explanatory scales describe the rate of mobility within the percentage of moving spermatozoa in the field of view [7]. The evaluation of the percentage of these mobile cells is as follows; 0 = immotile, and motile are 1=25%, 2=50% motile, 3=75% motile, 4= 75% and up of 4 [8]. All these nonlinear scales and arbitrary data cannot be used for statistical analysis. However, hemocytometric and spectrophotometric methods are followed from classical methods when spermatozoa concentrations are determined [9,10].

1.3. What is the importance of CASA systems in fish spermatology?

In general, the sperm cells of teleost fishes (especially in marine fish species) are fully immobile until they encounter external aquatic environment or female fish ovarian fluid [11]. In most teleost fishes with external fertility strategies, sperm activity is short (20-25 seconds in trout, especially 1-2 minutes in carp) and tends to decrease during motility period or after activation [12]. This occurs due to ATP consumption and deterioration of plasma membrane permeability in spermatozoon, and it is resulted in loss of fertilization ability [13,14]. It has become a necessity to develop methods that have fast, practical and reproducible properties in the determination of the quality parameters of fish spermatozoa with very short life span characteristics. For this reason, computer-assisted sperm analysis systems (CASA) developed for mammals but now it begun to be explored for use in fish.

Computer-assisted sperm analysis systems are very popular for the practical analysis of many living sperm kinematics and have taken the methods of predicting the analyzer's individual emotions with classical methods used in the past. CASA systems have become very important systems to determine low motility durations in especially fish sperm. The motility parameters such as VSL; straight line velocity (μm/s), VCL; curvilinear velocity (μm/s), VAP; angular path velocity (μm/s), LIN; linearity (%), VSL/VCL*100, BCF; beat cross frequency (cross/second) (Hz), ALH; amplitude of lateral displacement of the spermatozoon head (μm) and MAD; mean angular displacement (^{o}) can be determined in a very short time [1,15].

1.4. Gauss Filter

In BASA-Sperm Aqua Software, The Gaussian filter was used to remove sharp color transition on the image. Thus, video frame was smoothed and the detection of sperm cells has become more possible. Sperm video frames include two-dimensional pixel matrices. In a two-dimensional plane, Gaussian filter is expressed by the formula in Eq. (1) [16,17]:

$$g(x,y) = \frac{1}{2\pi\sigma^2} e^{\frac{-(x^2+y^2)}{2\sigma^2}} \quad (1)$$

In equation (1), the function g gives the Gaussian equivalent of the x and y coordinates. The σ value is the standard deviation of the Gaussian distribution, which will determine the sharpness or smoothness of the pixel at (x, y) coordinates. $1/2\pi\sigma^2$ is the normalization coefficient.

1.5. Otsu (Adaptive Thresholding)

In BASA-Sperm Aqua, Otsu adaptive thresholding was used to separate the sperm from the background within black and white color space. Otsu is an algorithm that automatically calculates the threshold value according to the pixels on the image. The algorithm assumes that there are two separate classes on the image and tries to find the maximum value of the variance between these two classes. The variance calculation for an N-length array is shown in Eq. (2) [18].

$$\sigma^2 = \sum_{i=0}^{N} (x_i - \bar{x})^2 Pr(x_i) \quad (2)$$

Variances between classes (black-and-white classes) are found by the formula in Eq. (3).

$$\sigma_w{}^2(t) = w_1(t)\sigma_1{}^2(t) + w_2(t)\sigma_2{}^2(t) \quad (3)$$

In Eq. (3) w is the likelihood of two classes separated by the t threshold value and the σ variance. Otsu specifies the class for each pixel by calculating the probability.

1.6. Circular Hough Transform

The Hough transform is an effective method for extracting properties of specific points known shapes. The Circular Hough Transformation (CHT) is also a feature extraction technique that is often used in the detection of round or ellipsoidal objects. In terms of circularity, CHT is a special version of Hough transformation. The most important advantage of CHT is that it can easily distinguish objects that are likely to be circles or have a radius in the image. In BASA-Sperm Aqua software, segmentation was performed by Circular Hough Transformation for sperm cell detection, since sperm heads have a circular or elliptical shape [19].

1.7. VIBE Algorithm

In the BASA-Sperm Aqua software, the VIBE algorithm was used for background subtraction of moving targets. The VIBE algorithm is written in C programming language and can be embedded in CPU, GPU and FPGA [20]. Thus, it can work without dependency on the hardware platform. This is one of the main reasons why the VIBE algorithm is preferred in BASA-Sperm Aqua software. Background removal techniques have to deal with three things in order to be successful:

(1) What is the model and how does it behave? 2) How was this model launched? 3) How to update this model over time? VIBE answers all of these questions successfully [20,21].

1.8. Kalman Filter

After background subtraction from sequential frames, the Kalman filter was used to estimate the state of moving object in the previous frame [22]. The Kalman filter is basically developed for signals related to linear systems. These signals are one-dimensional and real-time signals distorted by white Gaussian noise at the same time. The Kalman filter approach has provided the most appropriate sequential solution to the linear filtering problem. To compute the sequential solution, the updated estimate of the state, the previous state estimate, and the new input data are used. Therefore, it is sufficient to keep only the previous state estimate.

1.9. Hungarian Algorithm

In this study, the solution of the conflict problems encountered in tag assignment to moving targets was solved by the Hungarian algorithm [23]. Hungarian algorithm is solved the assignment problem for a *2n*-vertex bipartite graph by using $O(n^3)$ arithmetic operation. Assignment problems are the most common problems in motion analysis research. In the assignment problem, there are k targets and k different agents to reach these targets. There is cost of c_{ij} when the i^{th} agent reaches to j^{th} target. It is desirable to create an assignment plan with the lowest total cost provided that an agent is unconditionally assigned to each target and an agent is assigned to only one target.

1.10. Lagrange Interpolation

The path information of moving targets is calculated using (x, y) coordinate matrix obtained at discrete time frames. Discrete-time calculations may not produce accurate results when using devices with low Frame per Second (FPS) values. Polynomial approximated interpolation can be used to solve this problem. In BASA-Sperm Aqua software, Lagrange interpolation is used to approximate the value of the sperm path lines in continuous time [24].

With a Lagrange interpolation, a line or curve passing through the point of x_i and y_i is fitted. The path length is calculated by the polynomial formed by the fitting process. The polynomial grade varies with the number of points. The polynomial grade to be fitted for n points is $n - 1$. This can be seen as a disadvantage as the number of process increases. But it can be ignored to calculate the true path length. In Fig. 1, a line fitted from two points is shown. In Fig. 2, a curve is fitted from three points by Lagrange Interpolation.

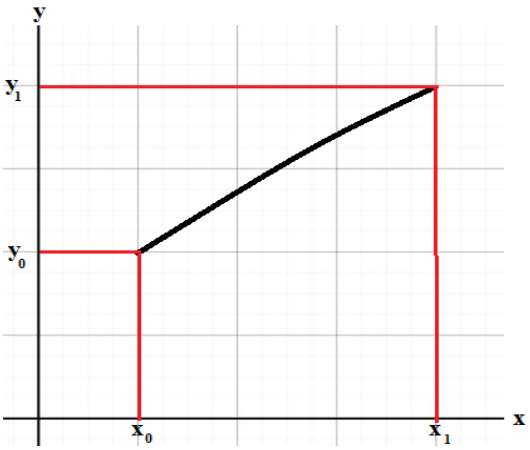

Figure 1. A line fitted from two points

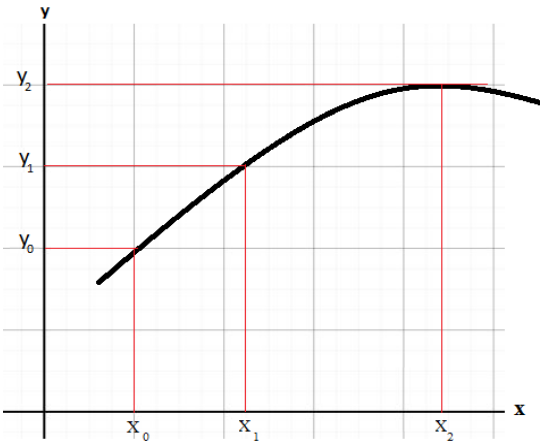

Figure 2. A curve is fitted from three points

The definition of Lagrange's polynomial approach is as follows: If a cluster contains k + 1 data, points are defined as;

$$(x_0,y_0),(x_1,y_1),\ldots,(x_j,y_j),\ldots,(x_k,y_k) \quad (4)$$

The Lagrange counterpart of the discrete points is as in Eq. (5).

$$L(x)=\sum_{j=0}^{k} y_j l_j(x) \quad (5)$$

In Eq. **Hata! Başvuru kaynağı bulunamadı.**, $l_j(x)$ base polynomial is computed as in Eq. **Hata! Başvuru kaynağı bulunamadı.**.

$$l_j(x)=\prod_{\substack{0\le m\le k\\ m\neq j}} \frac{x-x_m}{x_j-x_m}=\frac{x-x_0}{x_j-x_0}***\frac{x-x_{j-1}}{x_j-x_{j-1}}\frac{x-x_{j+1}}{x_j-x_{j+1}}***\frac{x-x_k}{x_j-x_k} \quad (6)$$

In Eq. **Hata! Başvuru kaynağı bulunamadı.**, since all x_j are different from each other, $x_j-x_m\neq 0$ at all times, and the equation will work perfectly.

2. Experimental Methods

2.1. The collection and preparation of sperm samples

The 3+ old rainbow trout (*Oncorhynchus mykiss*) used in this study was obtained from Inonu University Sürgü Vocational High School, Fish Reproduction and Research Unit, Malatya, Turkey, on February 2016. Semen samples were collected by stripping from fish. When the samples are taken, care is taken not to contaminate urea and feces. All semen samples were diluted with extender solution (ES, ratio 1:100, Semen:ES) and activated with activation solution (AS, ratio 1:20, Semen:AS) under microscope.

Extender Solution (ES): 7.60 g of NaCl, 2.98 g of KCl, 0.37 g of CaCl2 ・2H2O, 0.31 g of MgCl2 ・6H2O, 0.21 g of NaHCO3, and 1000-ml of distilled water (pH=8 adjusted with 1 N-NaOH) [25].

Activation Solution (AS): 7.5 g of NaCl, 0.2 g of KCl, 0.2 g of CaCl2 ・2H2O, 0.02 g of $NaHCO_3$, and 1000 ml of distilled water; pH: 8 [25].

2.2. BASA-Sperm Aqua Software

In BASA-Sperm Aqua software, all sperm samples motility parameters was investigated with Olympus CX 31 trinocular phase contrast microscope, 20x1,25 magnification lens, Sony CCD camera. The software of BASA-Sperm Aqua was newly developed CASA system for fish and aquatic creatures and its algorithmic flow is specific. The software was developed using C++ (for image processing) and C#.NET (for user interfaces) programming language on Microsoft Visual Studio IDE. The pseudo code and the algorithmic flow chart of BASA-Sperm Aqua were shown in Fig.3, and Fig.4, respectively.

```
1. Begin.
2. i = 0, j = 0 and spermCount =0
3. N = frame number for analysis
4. M = 0
5. For i = 0 to N do
6.      img = Capture  frame(i) in video
7.      img = Gauss(img) // smooth frame
8.      img = AdaptiveThreshold(img) //feature extraction
9.      hough = Hough(img)  // detect sperm.
10.     spermCount = spermCount + hough.size() // keep number of sperm
11.     If  i > 0 then // No action on first image
12.             img = BacgroundSubstruction(img)
13.             sperm = Kalman(img)  // estimate sperm cells
14.             sperm = Macar(sperm)  // solve assigning problems
15.             M = Max(sperm.number) // keep the largest number given for each sperm.
16.             For j = 0 to sperm.size() do
17.                     spermArray[i,j] = {i, sperm[j].number, sperm[j].XCoordinat, sperm[j].YCoordinat}
18.             End
19.     End
20. End
21. Average number of sperm =  spermCount / (i+1)
22. VSL = ComputeVSL(spermArray)
23. VCL = ComputeVCL(spermArray)
24. VAP = ComputeVAP(spermArray)
25. ALH = ComputeALH(spermArray)
26. MAD = ComputeMAD(spermArray)
27. STR = VSL / VAP
28. LIN = VSL / VCL
29. WOB = VAP/VCL
30. Classification of motility of sperm cells.
31. End.
```

Figure 3. The Pseudo Code of BASA-Sperm Aqua

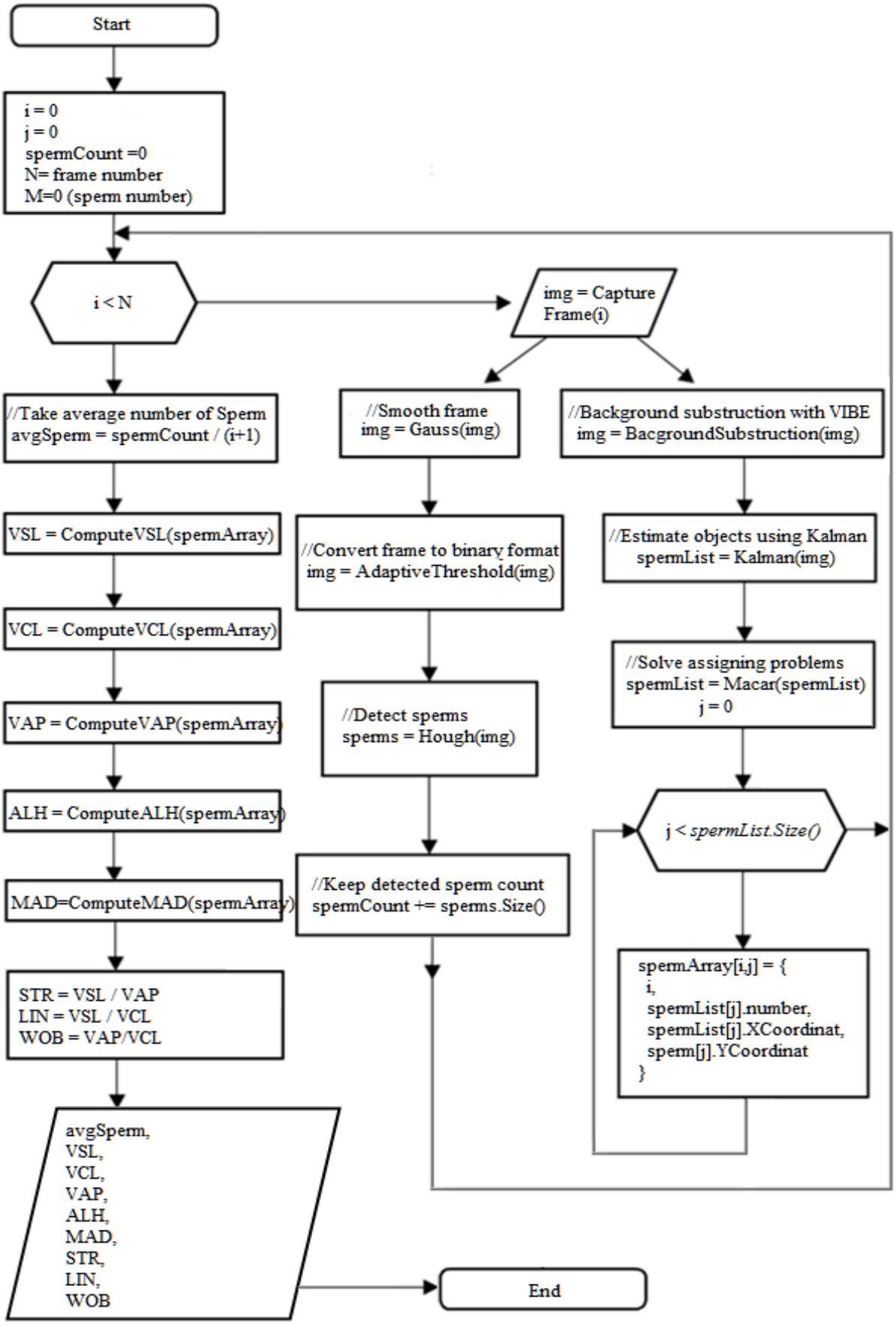

Figure 4. Flow chart of BASA-Sperm Aqua Software

An analog camera is used to display sperm videos mounted on a microscope. Using the camera APIs, the video was managed on the BASA Sperm Aqua software. The video recording interface of the analog camera is integrated into the BASA-Sperm Aqua software to transfer video to the computer. The image taken from the camera is automatically saved in a specified folder again with a specified name format. The hardware structure of BASA-Sperm Aqua has shown in Fig.5. All sperm samples motility parameters was investigated with Olympus CX 31 trinocular phase contrast microscope, 20x1,25 magnification lens, Sony CCD camera and computer aided sperm analysis system (BASA-Sperm Aqua) developed by Merk Biotechnology Limited Company, From Turkey.

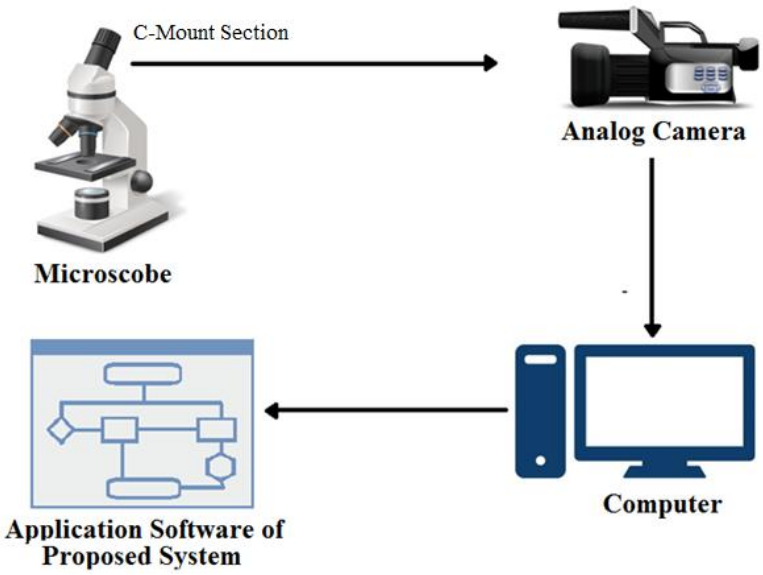

Figure 5. Hardware Structure of the BASA-Sperm Aqua

Figure 6 shows the video analysis interface of sperm cells. Recorded videos are analyzed in this interface using Algorithm 1. At the end of the analysis, quality parameters and analysis summary of sperm cells are presented. In this interface, new analysis, opening a recorded analysis and video cropping can be done.

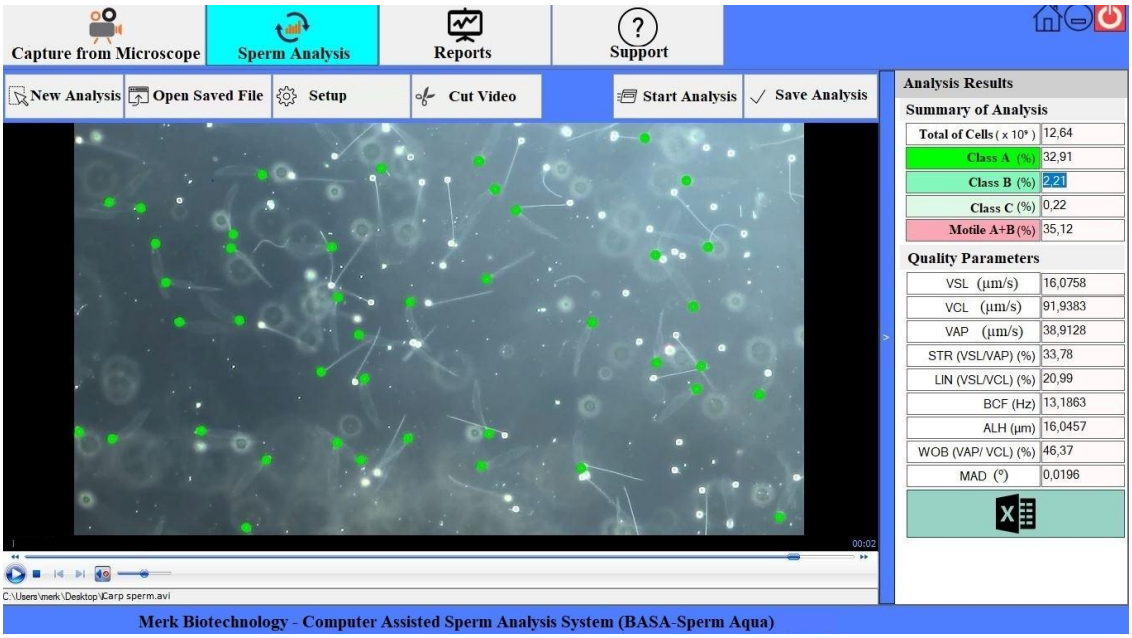

Figure 6. Video Analysis of Sperm Cells

BASA-Sperm Aqua can also make comparison of multiple analyzes statistically. Each analyzes can be recorded and compared in terms of the analysis parameters in the reporting interface screen. An example reporting screen is shown in Fig. 7. SPSS 17 program was used for statistical evaluation. Homogeneity was tested with the data from each individual Test of Homogeneity of Variances. The minimum and maximum values of the data, arithmetic mean and standard deviation (Mean±SD) were calculated. The graphics were created in Microsoft Excel.

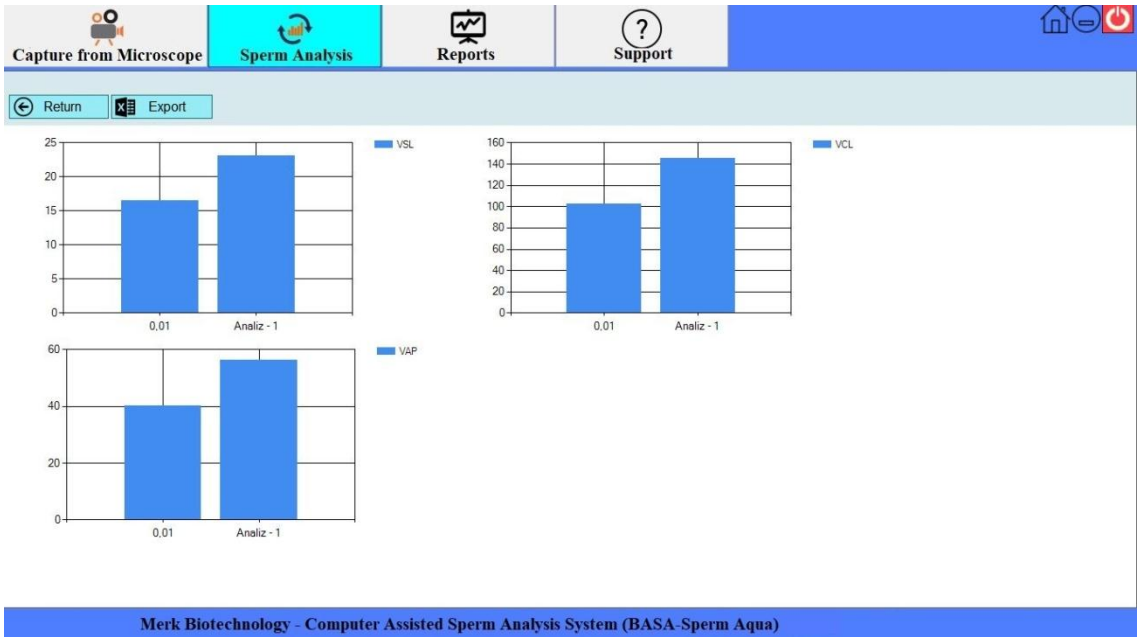

Figure 7. Reporting Interface for Comparative Statistics of Analyzes

2. Results and Discussion

This study was carried out with computer assisted analysis system to determine sperm quality in fish. For this purpose, the computer-assisted sperm analysis system (BASA-Sperm Aqua) which was developed first an indigenous product in Turkey was used. The sperm motility parameters of rainbow trout (*Oncorhynchus mykiss*) were found by the system (Table 1, Fig. 8).

Table 1. Motility parameters of rainbow trout spermatozoa

N=10	Mean±SD.	Min.-Max.
VSL (µm/s)	48.44±5.10	39.11-54.96
VCL (µm/s)	114.13±10.41	101.88 – 135.40
VAP (µm/s)	45.65±7.19	35.92-59.01
LIN (%)	19.74±8.17	10.07-33.83
BCF (Hz)	11.07±2.98	5.83-15.72
ALH (µm)	20.91±6.13	13.09-33.48
MAD (^{o})	0.03±0.01	0.02 - 0.06

In teleost fish, the spermatozoa generally have moved along a straight or slightly curved trajectory immediately after activation. Spermatozoa movement trajectories may turn into nested circles and increase in curvature at the end of the activation period, during exposure to contaminants, or in the unsuitable diluents. Under these conditions, the values of Linearity (LIN: the ratio of net distance moved to total path distance (VSL/VCL)) or the straightness (STR: the ratio of net distance moved to smoothed path distance (VSL/VAP)) can be very important determining parameters in determining the curvature of the orbit [26].

The spermatozoa of teleost fishes is immotile in the testes, initiation of movement but only when they come into contact with water, less than 2 min duration of movement and absence of acrosomes which are quite distinct from mammals. The two most important parameters in evaluating sperm quality are motility (active movement) and duration of movement. The spermatozoa orbits of fish are generally more curved than the mammals and fish sperm cells can move in 3 dimensions in a liquid medium [27]. However, the success of fertilization may depend on both the number of motile spermatozoa and their speed. Additionally, sperm motility (VSL or VCL) is a strong positive correlation with fertilization or the ration of egg hatching [26].

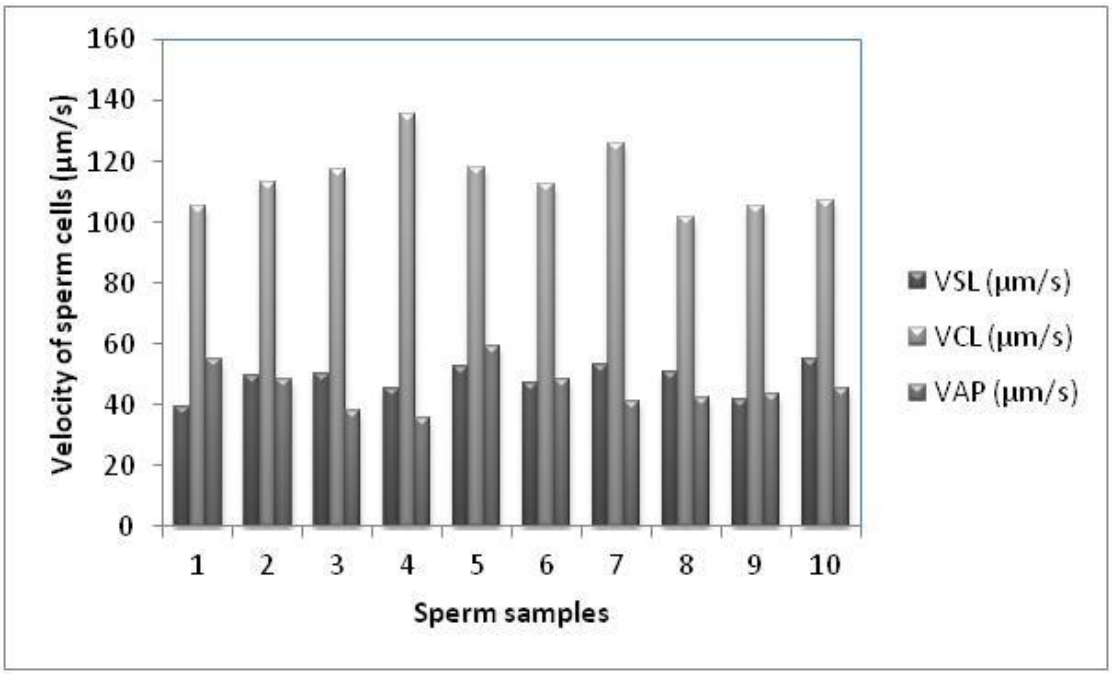

Figure 8. The VCL, VCL and VAP values of rainbow trout spermatozoa

Table 2. The Comparison between our results and literatures on motility parameters

Literature	Our results	Dietrich et al. (2005)	Boryshpolets et al. (2013)	Ciereszko et al. (2014)	Ciereszko et al. (2015)
			Mean±SD.		
VSL (µm/s)	48.44	38-45	56.8-84.1	45-50	39-40
VCL (µm/s)	114.13	73-83	105.4-128.6	145-155	95-110
VAP (µm/s)	45.65	-	88.4-115.3	95-100	65-70

According to our results, the value of VSL, VCL and VAP were different to other researchers (Table 2). The differences in the investigated values may be due to different computer assisted sperm analyzer systems [28], differences in the diet of fish [29], stress factors of mature fish [30], breeding season and age of mature fish [31].

3. Conclusions

As a result, the BASA-Sperm Aqua which is a sperm analyzer in newly designed to determine the quality parameters fish has been found to be a software that performs its functions very quickly and practical and produces accurate and understandable results. It is also thought that this software will enable the accumulation and storage of high quality sperm, especially in the aquaculture sector, by obtaining new and efficient individuals, and to enable the related scientific studies to be carried out with more real results.

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