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Araştırma Makalesi / Research Article

Comparison of Leja and Makler Chambers Performance in Rainbow Trout (*Oncorhynchus mykiss*) Sperm Quality in CASA System

Güneş YAMANER*[,a] Gökhan TUNÇELLİ[b] Momin MOMİN[c] Devrim MEMİŞ[d]
Istanbul Univ., Faculty of Aquatic Sciences, Department of Aquaculture and Fish Diseases, Istanbul, Turkey
*Corresponding author e-mail: gyamaner@istanbul.edu.tr
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ABSTRACT: In this study, the effect of the chamber used for the automated analysis of sperm motility and sperm kinematics parameters by CASA was evaluated of rainbow trout (*Oncorhynchus mykiss*) sperms. The assessment of motility parameters was carried out using CEROS II (Hamilton-Thorne, Beverly, MA, USA) connected to CX41 microscope (Olympus, Japan) at room temperature. Sperm samples were collected from five adult males by abdominal massage during the reproduction season and analyzed with two different chambers as follows specialty: Leja 2 cell chambered with 20 µl deep (Leja Products, Netherlands) and Makler chamber, round shape with 10 µl deep (Sefi-Medical Instrument, Haifa, Israel). Total sperm motility (Mot, %), and Velocity of Curvilinear (µm/s) were measured. For fertilization test, eggs from one female (550 g approximately 7500 eggs) were separated in equal five parts. Each part of eggs (approximately 1.500 eggs) was fertilized with each analyzed sperm (5ml). Fertilization, incubation procedure and calculation of fertilization rates have been kept as used routinely for rainbow trout culture procedure. The fertilization rates were found >80% for all used males. The motility percentage of samples analyzed by Leja has been found higher 90% while by makler changed between 26-45%. There is significantly effect on different chambers used in this study to determining the motility percentage. The high stability results and matched the fertilization success were detected in Leja 2-chamber. Statistical study with motility percentage showed a significant difference between Leja and Makler chambers ($p<0,05$).

Keywords: CASA, Leja 2, Makler, Motility parameters, Fertilization rate, Rainbow trout

Gökkuşağı Alabalığının (*Oncorhynchus mykiss*) Sperm Kalitesinde Bilgisayarlı Otomatik Sperm Analiz Sisteminde (CASA) Leja ve Makler Lamlarının Performansının Karşılaştırılması

ÖZ: Bu çalışmada gökkuşağı alabalığının (*Oncorhynchus mykiss*) spermine ait motilite ve motiliteye ait kinematik parametrelerin Bilgisayarlı Otomatik Sperm Analiz Sistemi (CASA) ile incelenmesinde farklı lamların kullanılmasının sonuçlar üzerindeki etkisi araştırılmıştır. Motilite ve parametreleri, oda sıcaklığında CEROS II (Hamilton-Thorne, Beverly, MA, USA) yazılım sistemine bağlı ışık mikroskobu (CX41, Olympus, Japan) ile incelenmiştir. Sperm örnekleri beş erkek balıktan abdominal masaj yöntemi ile balıkların üreme döneminde toplanmış ve Leja 2 (20 µl deep, Leja Products, Netherlands) ve Makler (10 µl deep, Sefi-Medical Instrument, Haifa, Israel) olmak üzere iki farklı lam kullanılarak analiz edilmişlerdir. Sperm örneklerinde toplam motilite (%), ve hız parametrelerinden eğrisel hız olarak ifade edilen VCL (µm/s) analiz edilmiştir. Dölleme çalışması için, bir dişi balıktan alınan yumurtalar (toplam 550 g, 7500 yumurta) beş eşit parçaya bölünmüştür. Bölünen yumurtaların her bir bölümü (1500 yumurta) analize tabi tutulan sperm örnekleri (5 ml) ile döllenmiştir. Dölleme, inkübasyon ve döllenme oranı prosedürleri, alabalık yetiştiriciliği için kullanılan rutin uygulama prosedürü altında gerçekleştirilmiştir. Döllenme yüzdesi her yumurta grubu için >%80 olarak bulunmuştur. İncelenen sperm örneklerinde Makler lamın kullanılması ile sperm motilite değerleri %26-45 arasında değişiklik göstermişken; Leja 2 lamı kullanılarak incelenen sperm örneklerinde her bir balıkta motilite değeri %90 ve üzeri bulunmuştur. Analiz esnasında iki farklı lam kullanılmasının motilite sonuçlarının belirlenmesini etkilediği bulunmuştur. Motilite sonuçları ve dölleme sonuçları karşılaştırıldığında ise Leja 2 lamı ile incelenen örneklerin motilite sonuçları döllenme oranı ile örtüştüğü ve daha kararlı sonuçlar verdiği görülmüştür. Motilite sonuçları ile yapılan istatistiksel değerlendirmede Leja 2 ve Makler lamlarından elde edilen sonuçlar arasında anlamlı bir farklılık tespit edilmiştir ($p<0.05$).

Anahtar Kelimeler: CASA, Leja 2, Makler, Motilite parametreleri, Döllenme oranı, Gökkuşağı alabalığı

INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is a commercially important culture species in Turkey. Selection of broodstock and knowledge about gametes quality are the most important factors in the aquaculture industries. The classification of gametes as "poor" or "good", in the other words, the

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[a]ORCID: https://orcid.org/0000-0003-1886-4985 [b]ORCID: https://orcid.org/0000-0003-1708-7272
[c]ORCID: https://orcid.org/0000-0001-5287-9537 [d]ORCID: https://orcid.org/0000-0003-2616-3601

determination of its ability to fertilize an egg is necessary before any experimental study to avoid the loss of effort, time and money. Fish eggs quality can be classified by morphological or macroscopic parameters. However, sperm quality, which can be defined as its ability to fertilize an egg successfully, has to be investigated under microscopic techniques. The traditional analysis of sperm sample includes the assessment of concentration, morphology of sperm cells and motility. The motility is one of the most important and basic tool to evaluate the quality of sperm which determines the fertility of the male individuals (Chong et al., 1983; Bromage and Roberts, 1995; Cabrita et al., 2008; Mananos et al., 2008). Prior to the development of computer-assisted sperm analysis (CASA) system, the most common way to evaluate sperm quality was based on subjective observations; sperm samples were classified using the number of motile sperm cells subjectively by 5 scale method under a light microscope. With the introduction of CASA system at the beginning of the 1980s, together with motility, several important parameters such as curvilinear, straight line and average path velocities, which are correlated to reproductive success in male, have been determined as the parameters of good quality sperm (Rurangwa et al., 2004).

The CASA system makes it possible to automatically view fields of sperm, get more detail, record kinematic parameters of sperm motility and storing information. Additionally, the data recorded by CASA is available for comparison of result and it makes possible to find delicate differences between individuals or treatments (Verstegen et al., 2002; Rurangwa et al., 2004; Caldeira and Soler, 2018).

Although CASA system assisted to take more accurate results than traditional methods, sperm quality assessment is also sensitive due to the numerous factors. As unrelated to the sperm samples; optical microscope, video camera, technician, software settings, frame rate, the number of fields analyzed, dilution rate and/or solution and type of chamber used for analysis could affect the motility results. CASA system technology needs to use the particular counting chambers and there are various types of chambers are available in the market which differs in terms of volume, depth, shape and loading method (Rijsselaere et al., 2003; Contri et al., 2010; Castellini et al., 2011; Gallego and Asturiano, 2018, 2019).

In a previous research, the effect of the type of chamber used has been studied human sperm (Le Lannou et al., 1992; Peng et al., 2015) and some animal species such as bulls (Contri et al., 2010; Lenz et al., 2011; Gloria et al., 2013; Ibănescu et al., 2016), boar (Gączarzewicz, 2015), rams (Palacín et al., 2013), horses (Hoogewijs et al., 2012), goat (Del Gallego at al., 2017); rabbits (Massányi et al., 2008) and dogs (Iguer-Ouada and Verstegen, 2001). The possibility of using different chambers may hinder the identification and quantification of factors potentially affecting CASA out-comes, and it is also necessary to harmonize and standardize laboratory procedure to use during CASA assessment for each species.

Therefore, the aim of the study to determine the effect of two chambers currently available on the market on rainbow trout sperm characteristics and to determine whether the CASA results may significantly affect the fertilization rate.

MATERIAL AND METHOD

Broodstock handling and gametes collection

Gametes were obtained from the rainbow trout broodstock at Sapanca Inland Fish Water Production, Research and Applied Station of Istanbul University, during spawning season (December, 2017). Five males and one female (3^+ years old) (body weight from 3 to 4 kg, (3608±306 g, total length from 30-40 cm (35.5±2.7 cm) were cultured in the concrete pond. Fish, both male and female, were fed daily with the commercial pellet diet for Salmonid. However, feeding was stopped two days before the experiment.

Sperm was collected from five males by gently hand-stripping in glass beaker taken to avoid contamination of blood, feces and urine. Eggs from one female that showed good morphology and color were used in fertilization. Eggs were obtained using abdominal massage directly into clean and dry egg container. Sperm samples were stored at 4 °C till the start of the analysis.

CASA system

The assessment of motility parameters was carried out using CEROS II (Hamilton-Thorne, Beverly, MA, USA) connected to CX41 microscope (Olympus, Japan) at 12 °C. Recordings were made with a digital camera (U-TV1X-2 Tokyo, Japan) at 60 images per second using the Rainbow trout variables predetermined in the Hamilton configuration. The following parameters were measured and analyzed as statistical: total sperm motility (Mot, %), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), straightness (STR, as VSL/VAP), linearity (LIN, as VSL/VCL) in each sperm samples. Sperm with velocity <20 μm s^{-1} were considered immotile, with velocity >20 μm s^{-1} were defined as motile. Due to unequal depth of viewing chamber, the focus knob was used to focus on the fluorescent sperm found in each viewing chamber.

Chambers and sperm motility evaluation

In experiment, two commercial types chambers were used as follows specialty: Leja 2 cell

chambered with 20 µl deep (Leja Products, Netherlands) and Makler chamber, round shape with 10 µl deep (Sefi-Medical Instrument, Haifa, Israel). The dilution rate was 1:250 (sperm:activator) for both chambers, and hatchery water (11±0.03 °C min 11- mak 11.05 °C) from tanks in which the fish were kept was used as an activator. In order to understand the relationship between fertilization rate and total motility parameters depending on the different chamber applications of CASA system, progressive motility result was discarded.

All motility analyses were performed in triplicate for each sperm sample using both chambers. Sperm analysis was conducted by the same operator in order to minimize error.

Fertilization experiment

Eggs from one female (550 g approximately 7500 eggs) were separated in equal five parts. Each part of eggs (approximately 1.500 eggs) was fertilized with each individual sperm (5ml). The sperm and eggs were gently stirred for 15-20 seconds. Then, 250 ml of hatchery water was added. In order to completion of hydration and swelling, the eggs were left for 30 minutes. Then the eggs were rinsed and transferred to separate baskets and incubated at 11 °C. Dead eggs were removed daily using a siphon. The fertilization success was determined as percentage of eyed-eggs (16 days after insemination) and calculated as (number of eyed eggs x initial egg number $^{-1}$*100%). In fertilization experimental design, the purpose of the use of eggs from a single female gives the opportunity to identify the motility parameters of each male individual by eliminating the variations in fertilization rates related to egg quality.

Data presentation and statistical analysis

All analyses were carried out with STATISTICA Software (StatSoft v.8). All results of spermatological parameters were analyzed using the Student's t-test. Results were considered statistically significant at a level of 0.05. The results are shown as mean ± SD.

RESULTS AND DISCUSSION

The mean values and the standard deviation of different types of chambers used for determination of sperm samples in rainbow trout are shown in Table 1. The average motility percentage of sperm samples were determined as 42.3±15.3%; 26.8±8; 39.1±1; 43.4±12.5 and 29.9±2.6 respectively for male 1;2;3;4 and 5 from Makler chamber. In Leja chamber, average motility percentage were 98.5±1.45%; 98.8±1.6%; 97.6±1.17%; 98.9±1,7% and 99.1±0.7% for male 1; 2; 3; 4 and 5 respectively (Table 1). The sperm samples analyzed using the Leja chamber showed considerable higher values in motility percentage. In addition, examined sperm samples used only with Makler, there is a significant difference between motility percentage result of individuals while Leja results were not showed any significant difference. Table 1 shows data obtained the result of VCL of sperm samples. As it can be observed, the similar VCL values for both chambers were obtained. The differences between Leja and Makler were not statistically significant ($p>0.05$). No significant differences among sperm samples of males tested with Leja in VCL ($p>0.05$). On the contrary, statistical analysis detected significant differences in VCL values between sperm samples of five male's that analyzed with Makler chamber (Table 1).

Table 1. The mean percentages of motility (%) and VCL (µm/s) from sperm samples as determined by Hamilton-Thorne Computer-Assisted Sperm Analyzer (Hamilton-Thorne) with evaluations using two different chambers: Leja and Makler, and fertilization rate (Mean±SD)

Individuals	Chambers	Motility (%)	VCL (µm/s)	Fertilization (%)
1	Leja	98.5±1.4^{a*}	132.6±3^{a}	85.6
	Makler	42.3±15.3^{b}	122.6±9.3 ^{b}	
2	Leja	98.8±1.6^{a}	132.3.±2.7^{a}	83.3
	Makler	26.8±8^{b,c}	114.4±4.5 ^{b,c}	
3	Leja	97.6±1.17^{a}	133.13±3.1^{a}	85.2
	Makler	39.1±1^{b}	122.6±10.2 ^{b}	
4	Leja	98.9±1.7^{a}	134.3±3.8^{a}	88.2
	Makler	43.4±12.5^{b}	120.3±3.7 ^{b}	
5	Leja	99.1±0.7^{a}	136.6±1.5^{a}	84.1
	Makler	29.9±2.6^{c}	102.±5.2 ^{c}	

*Means with the different superscript letter in same column are significantly different ($p<0.05$).

Fertilization rate were 85.6; 83.3; 85.2; 88.2 and 84.1% for male 1, 2, 3, 4 and 5, respectively. Based on the fertilization result, the type of chamber can affect the range of sperm motility and VCL values when controlled by the fertility male. The use of the Leja chamber resulted in significantly higher values for total motility, and according to the fertilization result, the repeated analysis of the sample resulted in high stability of the measures for two parameters (total motility and VCL) compared with Makler (Figure 1, Figure 2).

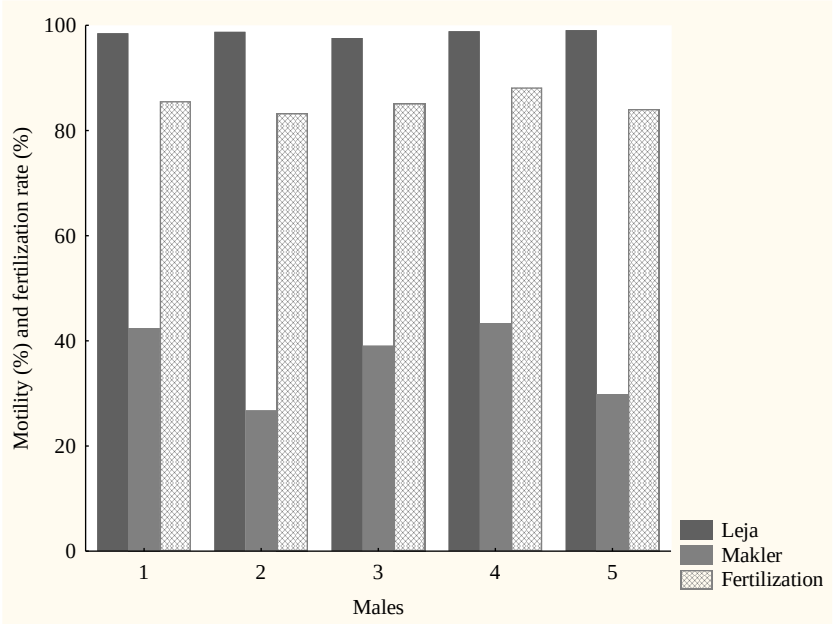

Figure 1. The motility percentage of sperm samples (%) and the fertilization rate

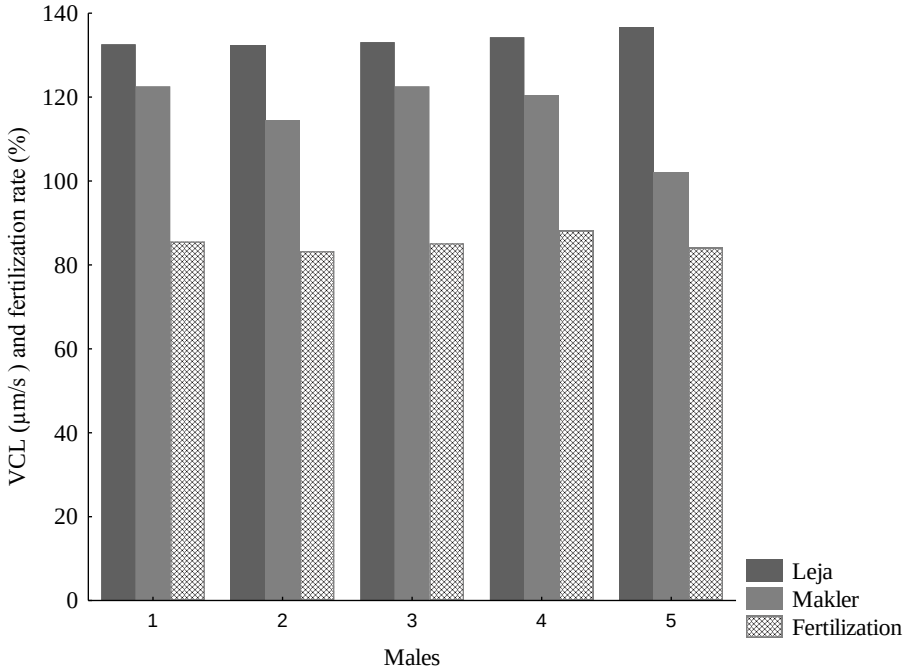

Figure 2. The VCL (µm/s) values of sperm samples and the fertilization rate

It is widely accepted that the motility is considered as a major important parameter for the evaluation of fresh sperm in fish species that give the fertilizing capacity of males. However, information

from scientific literature are unstable on this point; some studies confirm that fertilization capacity and sperm motility are interrelated (Rurangwa et al., 2004) while others suggest that this relationship is small or non-existent (Bobe and Labbe, 2010). Despite the fact CASA system considered in favor system for objective and repeatable assessment of motility and kinematic parameters than subjective method; these discrepancies may be due to lack of thorough standardization procedures for CASA assessment of sperm motility (Rijsselaere et al., 2003; Contri et al., 2010; Broekhuijse et al., 2012; Hoogewijs et al., 2012; Gloria et al., 2013; Palacín et al., 2013). The measurement of sperm motility could be affected by CASA system settings such as frame rate, the number of frames analyzed (Rijsselaere et al., 2003; Contri et al., 2010). An important effect that received detail attention recently, can be explained by the counting chamber type, which was reported in humans and in some animals species (Iguer-Ouada and Verstegen 2001; Massányi et al., 2008; Contri et al., 2010; Lenz et al., 2011; Hoogewijs et al., 2012; Gloria et al., 2013; Palacín et al., 2013). In this study, sperm motility determined by CASA in two different chambers Leja (capillary-loaded, 20 µm depth) and Makler (droplet-loaded, 10-µm depth) were related to the fertilization rate results as a reference.

In the present study, two different chambers types were used in order to understand the relationship fertilization rate and motility result of Rainbow trout sperm using CASA system.

This study clearly showed that the type of chamber significantly affects sperm motility characteristics. The number of motile cells detected was variable in two chambers. The motility percentage was highly repeatable and stable in Leja chamber, whereas it was less stable in Makler chamber according to fertilization rate. On the other hand, spermatozoa velocity was highly repeatable in all chambers, suggesting that this parameter was very stable in Leja chamber. That using different chamber affect motility has been established for the boar (Christensen et al., 2005), cattle (Contri et al., 2010; Lenz et al., 2011; Gloria et al., 2013), goats (Del Gallego et al., 2017), humans (Tomlinson et al., 2010; rabbits (Massányi et al., 2008), sheep (Palacín et al., 2013) and stallion (Jasko et al., 1990; Spizziri et al., 2010; Hoogewijs et al., 2012). Unlike our study, differences between Makler and Leja chamber have been concluded with the results showing that Makler chamber resulted in higher parameter values than obtained with Leja chamber in Ram sperm (Palacín et al., 2013) and goat (Del Gallego et al., 2017). However, other studies have not shown differences between different chambers, in both mammals (Gaczerzewicz, 2015) and fish (Gallego et al., 2013).

Two used chambers in this study have different depth and each fish species has different swimming specialty. This knowledge makes important to characterization of current fish spermatozoa. During the motility analysis, it is necessary to consider the chamber depth, by probably determining the way spermatozoa move in it, may contribute to differences between the motility results recorded by CASA for the sample evaluated in layers of different depth (20 µm vs 10 µm). For this reason, both in terms of CASA setting and in the use of different counting chambers in combination with CASA system literatures reported wide variety sperm analysis result (Cabrita et al., 2014).

The fertilization rates were found >80% for all used males. The motility percentages of samples analysed by Leja has been found higher 90% while by Makler changed between 26-45%. There is significantly effect on different chambers used in this study to determining the motility percentage. The high stability results and matched the fertilization success were detected in Leja 2-chamber. Statistical study with motility percentage showed a significant difference between Leja and Makler chambers ($p<0,05$). Although the Makler chamber is preferred due to the economic and ergonomic specialty, the using of Leja resulted in more accurately according to fertilization result and Makler chamber should be improved technically and practically.

In conclusion, previous reports showed that the chambers used in the CASA system gave varying results on the sperm motility of different fish species and it depends on the fish species, the technique used and the technician. Generally, it is difficult to choose the best one. But according to the results of this study, Leja chamber gave more realistic motility results which are very closely supported by fertilization results, however, Makler chamber gave irrelevant motility results in rainbow trout sperm. Sperm motility characteristics of each species may show differences and considering these differences, the most appropriate motility determination method should be developed and standardized for each species under specific experimental or practical conditions.

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Statement of Conflict of Interest

No potential conflict of interest was reported by the authors.

Authors' Contributions

GY and GT designed the research. GY, GT and MM did stripping and fertilization studies. GY and GT runned the CASA studies. GY made the statistical analysis. GY, GT, MM and DM wrote the article. All authors have read and confirmed the lastest version of the article.

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