Standardization of sperm motility analysis by using CASA-Mot for Atlantic salmon (*Salmo salar*), European eel (*Anguilla anguilla*) and Siberian sturgeon (*Acipenser baerii*)

Carina Caldeira[1,2,*], Sandra Hernández-Ibáñez[2], Anthony Valverde[2,3], Patrick Martin[4], Juan G. Herranz-Jusdado[5], Víctor Gallego[5], Juan F. Asturiano[5], Borys Dzyuba[6], Martin Psenicka[6], Carles Soler[1,2]

[1]PROISER R+D, Av. Catedrático Agustín Escardino, 9, Building 3 (CUE), Floor 1, 46980 Paterna, Spain.

[2]University of Valencia, Faculty of Biological Sciences, Campus Burjassot, C/ Dr Moliner 50, 46100 Burjassot, Spain.

[3]Technological Institute of Costa Rica, San Carlos Campus, School of Agronomy, 223-21001 Alajuela, Costa Rica.

[4]Conservatoire National du Saumon Sauvage, Larma, 43 300 Chanteuges, France.

[5]Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n, 46022 Valencia, Spain.

[6]University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic.

* Corresponding author
Email address: carina.caldeira@proiser.com (C. Caldeira)
Phone numbers: +34 961196060
Postal address: PROISER R+D, Av. Catedrático Agustín Escardino, 9, Building 3, Floor 1, 46980 Paterna, Spain

Abstract

It is essential to define an optimized standard method to assess the fish sperm quality to minimize the differences between the results obtained by different laboratories. Only this optimization and standardization can make them useful from academia to industry. This study presents the validation of sperm motility assessment using a CASA-Mot system for three endangered diadromous fish species: European eel (*Anguilla anguilla*), Atlantic salmon (*Salmo salar*) and Siberian sturgeon (*Acipenser baerii*). To attain this goal, different technical and data processing methods were tested: 1) magnification lens (x10 and x20), 2) Spermtrack® reusable chambers (10 and 20 µm depth) and 3) different frame rates ($50 \geq FR \leq 250$). The results suggested that the sperm motility assessment for eel, salmon and sturgeon should be performed at 200, 250 and 225 frames s^{-1}, respectively. Moreover, to obtain a high number of analysed spermatozoa in less time and a natural movement of the sperm cells, it is recommended to use x10 objective and 20 µm depth. In conclusion, different technical settings influence sperm kinetic parameters and should be validated for each fish species to allow the comparison of results between laboratories.

Keywords: Diadromous fish species; sperm quality; spermatozoa track; frame rate; counting chamber; magnification lens

1. Introduction

The marked decline of wild stocks of some diadromous fish species such as European eel (Jacoby and Gollock, 2014), Atlantic salmon (NASCO, 2016) and Sturgeon sp. (Ruban and Bin Zhu, 2010) due to construction of dams, pollution, poaching and overfishing, together with their economic importance and high commercial demand, aroused a great interest in their production in captivity. The efficacy of aquatic fertilization in captivity depends on the accurate evaluation of the sperm quality, which nowadays is the best way to define the fertility potential of each male (Kime et al., 2001; Rurangwa et al., 2004). For the assessment of sperm quality, it is needed to have available rapid and quantitative techniques as a useful tool for aquaculture purposes (Kime et al., 2001; Gallego et al., 2018a). Sperm motility is one of the most important parameters of sperm quality and is sensitive to biological and technical conditions during analysis (Rurangwa et al., 2004; Castellini et al., 2011).

Computer-assisted sperm analysis (CASA) is an accurate, reliable and objective technology which offer several spermatozoa quantitative parameters (Rurangwa et al., 2004; Caldeira et al., 2018). A complete CASA-Mot system, which is a CASA devoted to motility analysis (Soler et al., 2016; Holt et al., 2018), includes a software associated to a phase contrast microscope equipped with a video camera. However, in the market, there are a range of products or even different versions of the same product (Holt et al., 1994; Castellini et al. 2011). Besides the different CASA-Mot systems can follow the same general principle, each one has specific algorithms which can result in the incompatibility of results (Holt et al., 1994). This common principle consists in the individual measurement of spermatozoa motility based on the detection of spermatozoa head in consecutive images in order to obtain spermatozoa tracks (Mortimer et al., 1997; Bobé et al., 2010; Fauvel et al., 2010). In addition, the sperm quality assessment is also sensitive to the hardware systems, such as the optical microscope, video camera and

74 counting chambers (Castellini et al., 2011; Soler et al., 2012; Gallego et al., 2013; Del
75 Gallego et al., 2017; Bompart et al., 2018).
76 The frequency of images used on the motility analysis can be a limiting factor (Acosta
77 and Kruger, 1996) in the reconstruction of the trajectories and, consequently, some kinetic
78 parameters are frame rate (FR) dependent for both mammals and fish (Morris et al., 1996;
79 Castellini et al., 2011 Boryshpolets et al., 2013; Valverde et al., 2018). Therefore, it is
80 necessary to know the optimal frame rate that provides enough detail about spermatozoa
81 trajectory avoiding redundant information (Castellini et al., 2011). Sperm trajectory and
82 velocity can also be affected by counting chamber depth due to the natural movement of
83 spermatozoa (Kraemer et al., 1998; Bompart et al., 2018). This issue depends on the
84 different motility patterns, head shape and flagellum size and could be species-specific.
85 In this respect, a reliable and standardized method to analyse the sperm quality is needed
86 for each species. Thereby, it is important to enhance the reliability and comparability of
87 data provided by different research groups through the application of a standard
88 methodology for sperm analysis (Wilson-Leedy and Ingermann, 2007; Gallego et al.,
89 2013).
90 The aim of this study was to evaluate different technical settings such as frame rate,
91 counting chamber models and lens magnification to define a standard method for the
92 analysis of sperm motility of these three endangered fish species (*Anguilla anguilla*,
93 *Salmo salar*, *Acipenser baerii*) using a CASA system.
94

95 ## 2. Materials and methods

96 ### *1.1. Sperm sampling*

97 Sperm samples were collected from three fish species: European eel (*A. Anguilla*; n = 5),
98 Atlantic salmon (*S. salar*; n = 5) and Siberian sturgeon (*A. baerii*; n = 3). Mature males

were sampled during 2017 in different facilities, according to the reproduction season and the procedures specific to each species. Eel sperm samples were collected on March in the facilities of the Universitat Politècnica de València (Valencia, Spain; Herranz-Jusdado et al., 2018). Wild salmon males were sampled on November at the Conservatoire National du Saumon Sauvage (Chanteuges, France; Caldeira et al., 2018). Sperm samples of Siberian sturgeon were collected on May at the University of South Bohemia (Vodnany, Czech Republic; Psenicka et al., 2007). In all facilities, photoperiod and temperature were adjusted to simulate the natural environmental conditions of each species. Sperm samples were immediately transported to the laboratory and kept at 4°C until sampling and analysis.

Procedures involving animal subjects (Eel, Salmon and Sturgeon) have been approved for the three research institutions by the official organisation of each country (Spain, France and Czech Republic).

1.2. CASA-Mot analysis

Sperm motility was assessed by using the Integrated Semen Analysis System (ISAS®v1, PROISER R+D, S.L., Paterna, Spain), a CASA-Mot system that included a phase-contrast microscope (UOP; PROISER) connected to a video camera (MQ003MGCM; XIMEA, Münster, Germany), with an FR of 500 frames per second (fps) and a final resolution of 640x480 pixels.

Sperm motility was analysed using two reusable counting chambers with different depths (Spermtrack® 10 and 20 µm; PROISER), at magnifications x10 and x20 with negative phase contrast. Sperm samples were activated on the chamber by mixing a drop of ejaculate with a 2 or 4 µL (for Spermtrack® 10 and 20 µm, respectively) of the adequate activator medium for each species. Eel samples were activated with artificial seawater

with 2% BSA (Caldeira and Soler, 2018), whereas for salmon and sturgeon sperm samples were activated with distilled water. However, in case of sturgeon, 0.5% BSA were added to prevent sperm adhesion to the glass surface. Video recordings started 5 s post-activation, and each sample was recorded three times.

All semen samples were recorded at 500 fps for 1 s and then the videos were segmented into 50, 100, 150, 200 and 250 FR videos. The command used was: [*echo off: set fps= 50, 100, 150, 200: for %%i in (.*.avi) do (set fname=%%~ni) & call: encodeVideo; goto eof: encodeVideo: ffmpeg.exe -i %fname%.avi -r %fps% -c libx264 -preset slow -qp 0 "%fname%_(%fps%fps).avi"; goto eof*].

Total sperm motility (MOT; %), as well as several kinetic motility parameters (Bompart et al., 2018), were considered for this study: curvilinear velocity (VCL; μm s^{-1}), straight-line velocity (VSL; μm s^{-1}) and average path velocity (VAP; μm s^{-1}), linearity (LIN = VSL/VCL, %), straightness (STR = VSL/VAP, %), wobble (WOB = VAP/VCL), %), and beating measurements, such as amplitude of lateral head movement (ALH; μm) and beat-cross frequency (BCF; Hz). Software settings were adjusted for the sperm analysis of each species and the different FR.

1.3. Statistical analysis

The optimal FR for each species, depending on the other two technical categories (magnification lens and chamber), were obtained based on the nonlinear model $y = \alpha \exp(-\beta/x)$, where y corresponds to VCL and x the FR. The asymptotic level was represented by α, which is the maximum value when the FR is above the threshold level (calculated as the FR needed to obtain 95% of the maximum level); the rate of the approach to the asymptote was represented by β, which indicate the rate of increase of VCL as FR increases.

149 The data obtained from the analysis of some kinematic parameters (VCL, VSL, VAP,
150 LIN, STR, WOB, ALH, BCF) were first tested for normality and homoscedasticity using
151 the Shapiro–Wilk, normal probability plot, and Levene tests respectively. The generalized
152 linear model (GLM) procedure was used to evaluate the influence on the kinematic
153 parameters on the factorial ANOVA and significance of main effects of the lens (x10 and
154 x20), chamber (10 µm and 20 µm), interactions and for the FR optimal of each fish
155 species. Differences between means were analysed by the Bonferroni test. Results for the
156 percentage of motility and the kinematic parameters are presented as the mean ± standard
157 error of the mean (SEM). Statistical significance was set at P = 0.05 (two-sided). All data
158 were analysed using Statgraphics Centurion XVII, 17.2.04. (32-bit) (1982-2016 for
159 Statpoint Technologies, Inc., EE. UU.).
160

161 ## 3. Results

162 ### *3.1. General results*

163 The highest motility rate was found in sturgeon, whilst eel samples showed the lowest
164 motility. Independent of species, the FR had no effect on the motility rate considering
165 both magnification lens and chamber. However, some significant differences were
166 observed between lens and chamber within the same FR (data not shown). Otherwise,
167 other kinetic values were extremely affected by FR.
168 The most notable difference was registered in the sperm motility traits of each fish species
169 (Figure 1), with the catadromous species (European eel) exhibiting the lowest velocity
170 and straightness of motion of spermatozoa tracks than anadromous species (Atlantic
171 salmon and Siberian sturgeon). Eel sperm was the slowest with the lowest linearity,
172 whereas salmon were the fastest and the sturgeon had the highest linearity. However, the
173 behaviour of the kinetic parameters of the three diadromous fish species was similar

(Figure 2-4). There was a significant progressive increase in VCL as the FR increased. There were no significant differences in VSL for salmon and sturgeon, whereas the eel sperm showed statistical differences regardless of the technical conditions (magnification lens and chamber). Therefore, LIN decreased significantly as the FR increased in all three species.

3.2. Effect of frame rate

Eel sperm showed the lowest α (corresponding with the estimated optimal FR for the asymptotic threshold level) value for MOT, whereas sturgeon samples had the highest (Table 1). The optimal FR for MOT ranged from 41.92 to 61.56 fps for eel, 82.08 to 93.57 fps for salmon and 96.77 to 99.90 fps for sturgeon, depending on the technical conditions (lens and chamber). This means that the optimal FR for the analysis of motility rate could be 100 Hz for the three fish species, or even 75 fps can be also adequate for eel.

The sperm kinematic values of eel, salmon and sturgeon were dramatically affected by FR, although the threshold level was different for each species. Considering the VCL as the most sensitive parameter (Table 2), eel showed the lowest α value (188.88 to 203.08 fps), while salmon showed the highest asymptotic level (253.08 to 260.18 fps). Therefore, independent of technical categories, the considered optimal FR was 200 fps for eel, 225 fps for sturgeon and 250 fps for salmon. The correspondent setup for the optimal FR was minimum particle area of 3 µm for eel and 5 µm for salmon and sturgeon, and connectivity of 5 µm for eel and 6 µm for the other two species.

3.3. Effect of magnification lens and chamber depth

Attending the previous results, the effect of magnification and depth on motility was analysed at 100 fps for the three species. The different magnification lens tested at 10 µm

depth affect significantly the MOT of eel and salmon sperm, while sturgeon was not significantly affected by these technical conditions (Table 3). The interaction between magnification and chamber depth had no effects on motility rate for all the fish species studied (data not shown).

When considered the results obtained by the optimal FR for each species, several kinetic values were affected by both magnification and chamber depth technical categories (Table 4). Eel sperm showed significant higher VCL, VSL and VAP values for the x10 objective and 20 µm depth, while for sturgeon sperm that parameters were significantly higher for 20 µm depth tested under x20 objective. In the case of sturgeon, the magnification lens did not significantly affect the spermatozoa velocity. Salmon sperm had higher VCL and VAP in case of x10 objective tested in 10 µm depth chamber, although not significant differences between depths were observed. The other kinematic parameters had a similar trend for all fish species, showing the lowest LIN, STR, BCF and the highest WOB, ALH for x10 objective. However, the interaction of the technical conditions (magnification and chamber depth) at optimal FR showed an effect on different kinematic parameters among these fish species. Eel sperm had significant differences on the wobble coefficient (WOB), while in salmon and sturgeon sperm the effect was related with linearity (LIN and STR) and velocity (VCL), respectively (data not shown). Eel sperm showed significant differences on WOB tested in 10 µm depth chamber, showing significant higher values for x10 objective. At the same technical condition (x10 objective and 10 µm depth chamber), salmon sperm showed significant lower linearity. For sturgeon sperm, the velocity was affected by the chamber depth tested under x20 objective, being higher for 20 µm depth.

4. Discussion

Classical assessment of sperm quality was established following a subjective analysis based on the estimation of concentration and percentage of motility. This method introduces a great variability on the results (Rurangwa et al., 2004), reducing their reliability and, consequently, their biological significance and practical utility (Gallego et al., 2018b). For this reason, CASA systems were developed about 30 years ago (Bompart et al., 2018). A computerised system is considered an objective analysis that provides rapid, accurate and quantitative measurements of motility parameters producing a large amount of data (David et al., 1981; Verstegen et al., 2002; Didion, 2008; Björndahl, 2011). In the market, there are different CASA systems brands or even different versions of the same system. Unfortunately, the wide range of technical conditions and procedures used by different laboratories precludes the standardization and comparison of the results presented in the literature (Gill et al., 1988; Vantman et al., 1988; Jasko et al., 1990; Boryshpolets et al., 2013; Gallego et al., 2013; Sadeghi et al., 2017). For this reason, it is essential to define standard methods to assess the sperm motility for each species, based on the largest number of technical conditions (magnification lens, frame rate acquisition, depth of the chamber models, software settings, activation media, start time of measurements after sperm activation and total time of analysis) that can affect the results. Thereafter, it will be possible to minimize the differences between the results by different laboratories and to transfer them from academia to industry (Rurangwa et al., 2004; Gallego et al., 2018a). In this study, different technical settings were assessed in order to standardise the sperm quality evaluation of three threatened diadromous fish species (European eel, Atlantic salmon and Siberian sturgeon) and minimize these differences.

The basic principle of the CASA-Mot systems is the acquisition and analysis of successive images of motile spermatozoa. Up till now, most of the systems were using

low standard FRs (16, 25, 30, 50 or 60 fps) due to limitations of hardware and software (Holt and Warme, 1977; Stephens et al., 1988; Holt and Palomo, 1996; Morris et al., 1996; Castellini et al., 2011; Gallego et al., 2013; Parodi et al., 2015). However, it has been demonstrated in mammals that higher frame rate increases some velocity parameters, such as VCL, STR, BCF (Mortimer et al., 1988; Mortimer and Swan, 1995; Castellini et al., 2011). At lower FRs the analysed trajectory can underestimate the real value of kinetic traits, particularly for fast and nonlinear spermatozoa, whereas at higher FRs the information can arrive to become redundant (Mortimer and Swan 1999; Castellini et al. 2011). In this way, it is necessary to define the "optimal" frame rate to provide detailed and truthful information based on an accurate reconstruction of the spermatozoa trajectories (Castellini et al., 2011; Gallego et al., 2013; Bompart et al., 2018; Valverde et al., 2018). Therefore, this study showed for the first time the mathematical definition of the optimal FR based on videos captured at an FR of 500 fps and analysed at 250 fps for each species studied.

The study of both total and progressive motility percentages is commonly considered enough for the calculation of seminal doses production in most of the farmed mammals (Castellini et al., 2011; Valverde et al., 2018). Total motility was not affected by the FR in any of the three species studied here. This result is in accordance with that observed in species as the boar (Valverde et al., 2018), bull, man, rabbit and ram (Castellini et al., 2011). In any case, the optimal frame rate for the measurement of motility was established on 75 fps for eel and 100 fps for salmon and sturgeon.

Following the same behaviour described in other species (Castellini et al., 2011; Parodi et al., 2015; Valverde et al., 2018), in the three fish species studied here VCL was highly affected by FR. In opposition, no substantial affection of the VSL was observed, resulting in the LIN decrease. Our results corroborate previous studies (Contri et al., 2010;

Castellini et al., 2011; Boryshpolets et al., 2013; Gallego et al., 2013; Valverde et al., 2018), which suggested that the higher FR will generate the "real" spermatozoa trajectory. More sophisticated video cameras and computers are being continuously developed which improve the image acquisition at FRs previously impossible to reach. However, the maximum frame rate (up to 250 frames s^{-1}) currently available could be on the limit or even not be enough to work at the maximum sperm speed of some species. Fish spermatozoa are considered to have one of the fastest trajectories and, as it was possible to observe in this study, salmon were the species with higher sperm speed and an asymptotic level above 250 frames s^{-1}. For instance, it was also suggested that 290 frames s^{-1} is the FR required to fully trace the rabbit movement path (Castellini et al., 2011). This can imply that for some species could be necessary to increase the FR. Therefore, the FR variation is species specific and must be defined for each species to standardize the protocol and obtain reliable results (Mortimer et al., 1988; Mortimer and Swan, 1995; Castellini et al., 2011; Boryshpolets et al., 2013; Valverde et al., 2018).

The effect of the magnification lens on the sperm motility parameters can be explained by the different size of the analysed fields and, consequently, the final number of analysed cells. When the motility analysis is made at the highest magnification lens (x20) the lower number of spermatozoa that can be captured leads to a higher data variation and non-so accurate measurement of sperm parameters (Gallego et al., 2013). Following this principle, sturgeon sperm showed higher VCL for x20 objective, although the SEM was much higher than those obtained with x10 objective. On the contrary, eel and salmon sperm presented higher spermatozoa speed for results obtain with x10 objective, which was the data with less variation. Therefore, the motility analysis of eel, salmon and sturgeon sperm should be more accurate and precise using x10 objective.

Currently, there are available counting chambers based on two principles of microfluidic flows, capillarity and droplet displacement (Del Gallego et al., 2017; Bompart et al., 2018) that can be used for the analysis of spermatozoa motility using CASA-Mot systems. However, the assessment of fish sperm motility should be performed in the chamber charged by the second principle (reusable chambers), since the motility is dramatically affected by the time post-activation which limits the time of analysis. In addition, this kind of chambers are presented in different depths (10 and 20 µm) that can affect the spermatozoa movement. Fish spermatozoa are characterized by a large tail, being greater than the chamber depth, which means that the spermatozoa movement is restricted in the counting chamber and the cells could not reach the maximum speed (Hoogewijs et al., 2012; Soler et al., 2012; Bompart et al., 2018). In this study, eel and sturgeon spermatozoa reach higher speed with 20 µm depth (164.31 and 208.68 µm s^{-1}, respectively), whilst salmon spermatozoa showed the highest VCL for 10 µm depth (238.19 µm s^{-1}). However, in the last species, the WOB was significantly lower in the chamber with 20 µm. Thus, based on these results and on the fact that higher depth implies natural movement, the use of a chamber with 20 µm depth is recommended for these three diadromous fish species. The size and shape of spermatozoa could be so diverse among fish species that lead to a different sperm motility behaviour. However, the fluid resistance of the sperm head is lower than the sperm flagella, which means that the sperm movement results mainly from the interactions of flagellum with the surrounding medium (Baccetti et al., 1975; Vladić et al., 2002). Sperm flagellum has a microtubular structure, the axoneme, that contains many proteins and some of them are motor proteins that interact with microtubes as a source of energy for sperm motility (Baccetti et al., 1975; Brokaw, 1994). The dynein arms are ATPases that convert the ATP stored to produce mechanical work needed for bending behaviour of the flagella (Brokaw, 1994). Therefore, the length of the sperm

flagellum could be related to a high energy production that confers a fitness advantage (Vladić et al., 2002). Following this principle, the differences observed on the spermatozoa velocity of the three fish species can be explained by the flagellum size and axoneme organization. Salmon and sturgeon spermatozoa have a tail size under 40 µm (about 41-42 and 44 µm, respectively) with a typical 9 + 2 flagellar organization (Vladić et al., 2002; Psenicka et al., 2007), although the salmon have a sphere head and Siberian sturgeon an elongated spermatozoa head with acrosome. Eel spermatozoa have a curved and elongated head form with about 30 µm tail size that is organised in a 9 + 0 microtubular structure (Woolley, 1997; Marco-Jiménez et al., 2006). Thereby, the faster swimming sperm detected on males of anadromous species (salmon and sturgeon) may be correlated with the high storage of ATP in longer spermatozoa.

5. Conclusion

Computer-assisted sperm analysis systems are considered a valuable tool for quantitative analysis of sperm motility. At a practical level, this technique could be an indicator of high-quality breeders and can apply for the reproductive biology studies as well as for standard artificial insemination or assisted reproduction techniques for fish species (Gallego et al. 2018c). However, the optimization and standardization of the protocol at the technical level for each species is a fundamental requirement to make CASA-Mot a really useful tool not only to carry out studies about spermatozoa kinetic parameters but also to compare the results among different laboratories. In this study, the sperm motility assessment with different technical conditions suggested that the FR is the protocol variable that affects more the measurement of kinetic parameters and is species-specific. Therefore, the general recommendation for eel, salmon and sturgeon sperm analysis is 200 fps, 250 fps and 225 fps, respectively, combined with the use of x10 objective and a

counting chamber with 20 µm depth. In addition, our study suggested that the species with the longest spermatozoa have the fastest sperm.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No [642893]. AV is granted by the CONICIT and MICITT, Costa Rica. The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program), project Biodiversity (CZ.02.1.01/0.0/0.0/16_025/0007370), by the Czech Science Foundation (project No. 17-19714Y).

References

Acosta, A., Kruger, T.F., 1996. Human spermatozoa in assisted reproduction, 2nd ed. Parthenon Publishing Group Ltd, New York.

Baccetti, B., Bernini, F., Biglardi, E., Burnini, A.G., Dallai, R., Giusti, F., Mazzini, M., Pallini, V., Renieri, T., Rosati, F., Selmi, G., Vegni, M., 1975. Motility patterns in sperms with different tail structure, in: Afzelius, B.A. (Ed.), The Functional Anatomy of the Spermatozoon. Pergamon Press, Oxford, pp. 141–150.

Björndahl, L., 2011. What is normal semen quality? On the use and abuse of reference limits for the interpretation of semen results. Hum. Fertil. (Camb). 14, 179–186.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165, 535–548.

Bompart, D., García-Molina, A., Valverde, A., Caldeira, C., Yániz, J., Núñez de Murga, M., Soler, C., 2018. CASA-Mot technology: How results are affected by the frame rate and counting chamber. Reprod. Fertil. Dev. 30, 810–819.

374 Boryshpolets, S., Kowalski, R.K., Dietrich, G.J., Dzyuba, B., Ciereszko, A., 2013.
375 Different computer-assisted sperm analysis (CASA) systems highly influence sperm
376 motility parameters. Theriogenology. 80, 758–765.
377 Brokaw, C.J., 1994. Control of flagellar bending: a new agenda based on dynein diversity.
378 Cell Motil. Cytoskeleton. 28(3), 199-204.
379 Caldeira, C., García-Molina, A., Valverde, A., Bompart, D., Hassane, M., Martin, P.,
380 Soler, C., 2018. Comparison of sperm motility subpopulation structure among wild
381 anadromous and farmed male Atlantic salmon (*Salmo salar*) parr using a CASA system.
382 Reprod. Fertil. Dev. 30, 897–906.
383 Caldeira, C., Soler, C., 2018. Fish Sperm Assessment Using Software and Cooling
384 Devices. J. Vis. Exp. 137, e56823. doi:10.3791/56823.
385 Castellini, C., Dal Bosco, A., Ruggeri, S., Collodel, G., 2011. What is the best frame rate
386 for evaluation of sperm motility in different species by computer-assisted sperm analysis?
387 Fertil. Steril. 96, 24–27.
388 Contri, A., Valorz, C., Faustini, M., Wegher, L., Carluccio, A., 2010. Effect of semen
389 preparation on casa motility results in cryopreserved bull spermatozoa. Theriogenology.
390 74, 424–35.
391 David, G., Serres, C., Jouannet, P., 1981. Kinematics of human spermatozoa. Gamete
392 Res. 4, 83–95.
393 Del Gallego, R., Sadeghi, S., Blasco, E., Soler, C., Yániz, J.L., Silvestre, M.A., 2017.
394 Effect of chamber characteristics, loading and analysis time on motility and kinetic
395 variables analysed with the CASA-Mot system in goat sperm. Anim. Reprod. Sci. 177,
396 97–104.
397 Didion, B.A., 2008. Computer-assisted semen analysis and its utility for profiling boar
398 semen samples. Theriogenology. 70, 1374–1376.

399 Fauvel, C., Suquet, M., Cosson, J., 2010. Evaluation of fish sperm quality. J. Appl.
400 Ichthyol. 26, 636–643.
401 Feunteun, E., 2002. Management and restoration of European eel population (*Anguilla*
402 *anguilla*): an impossible bargain. Ecol. Eng. 18, 575–91.
403 Gallego, V., Carneiro, P.C.F., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Soler, C.,
404 Pérez, L., Asturiano, J.F., 2013. Standardization of European eel (*Anguilla anguilla*)
405 sperm motility evaluation by CASA software. Theriogenology. 79, 1034–1040.
406 Gallego, V., Asturiano, J.F., 2018a. Sperm motility in fish: technical applications and
407 perspectives through CASA-Mot systems. Reprod. Fertil. Dev. 30, 820–832.
408 Gallego, V., Herranz-Jusdado, J.G., Rozenfeld, C., Pérez, L., Asturiano, J.F., 2018b.
409 Subjective and objective assessment of fish sperm motility: when the technique and
410 technicians matter. Fish Physiol. Biochem. 1–11.
411 Gallego, V., Asturiano, J.F., 2018c. Fish sperm motility assessment as a tool for
412 aquaculture research: a historical approach. Rev. Aquacult. *in press*.
413 Gill, H.Y., Van Arsdalen, K., Hypolote, J., Levin, R., Ruzich, J., 1988. Comparative study
414 of two computerized semen motility analyzers. Andrologia. 20, 433–440.
415 Herranz-Jusdado, J.G., Kása, E., Kollár, T., Gallego, V., Peñaranda, D.S., Rozenfeld, C.,
416 Pérez, L., Horváth, Á., Asturiano, J.F., 2018. Handling and Treatment of Male European
417 Eels (*Anguilla anguilla*) for Hormonal Maturation and Sperm Cryopreservation. J. Vis.
418 Exp. 131, e56835. doi:10.3791/56835.
419 Holt, W., Watson, P., Curry, M., Holt, C., 1994. Reproducibility of computer aided semen
420 analysis: comparison of five different systems used in a practical workshop. Fertil. Steril.
421 62, 1277–82.

Holt, W.V., Palomo, M.J., 1996. Optimization of a continuous real-time computerized semen analysis system for ram sperm motility assessment, and evaluation of four methods of semen preparation. Reprod. Fert. Dev. 8, 219–230.

Holt, W.V., Cummins, J.M., Soler, C., 2018. Computer-assisted sperm analysis and reproductive science; a gift for understanding gamete biology from multidisciplinary perspectives. Reprod. Fertil. Dev. 30, iii–v.

Hoogewijs, M.K., de Vliegher, S.P., Govaere, J.L., de Schauwer, C., de Kruif, A., van Soom, A., 2012. Influence of counting chamber type on CASA outcomes of equine semen analysis. Equine. Vet. J. 44, 542–549.

Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E., Ollevier, F., 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 130, 425–33.

Jacoby, D., Gollock, M., 2014. *Anguilla anguilla*. The IUCN Red List of Threatened Species 2014: e.T60344A45833138.

Jasko, D.J., Lein, D.H., Foote, R.H., 1990. A comparison of two computer-assisted semen analysis instruments for the evaluation of sperm motion characteristics in the stallion. J. Androl. 11, 453–459.

Kraemer, M., Fillion, C., Martin-Pont, B., Auger, J., 1998. Factors influencing human sperm kinematic measurements by the Celltrak computer-assisted sperm analysis system. Hum. Reprod. 13, 611–619.

Liu, Y.T., Warme, P.K., 1977. Computerized evaluation of sperm cell motility. Computers Biomed. Res. 10, 127–138.

Marco-Jiménez, F., Pérez, L., Viudes de Castro, M.P., Garzón, D.L., Peñaranda, D.S., Vicente, J.S., Jover, M., Asturiano, J.F., 2006. Morphometry characterisation of European

446 eel spermatozoa with computer-assisted spermatozoa analysis and scanning electron
447 microscopy. Theriogenology. 65, 1302–1310.
448 Morris, A.R., Coutts, J.R., Robertson, L., 1996. A detailed study of the effect of video
449 frame rates of 25, 30 and 60 Hertz on human sperm movement characteristics. Hum.
450 Reprod. 11, 304–10.
451 Mortimer, D., Goel, N., Shu, M.A., 1988. Evaluation of the CellSoft automated semen
452 analysis system in a routine laboratory setting. Fertil. Steril. 50, 960–968.
453 Mortimer, S.T., Swan, M.A., 1995. Kinematics of capacitating human spermatozoa
454 analysed at 60 Hz. Hum. Reprod. 10, 873–879.
455 Mortimer, S.T., Schoëvaërt, D., Swan, M.A., Mortimer, D., 1997. Quantitative
456 observations of flagellar motility of capacitating human spermatozoa. Hum. Reprod. 12,
457 1006–1012.
458 Mortimer, S.T., Swan, M.A., 1999. Effect of image sampling frequency on established
459 and smoothing-independent kinematic values of capacitating human spermatozoa. Hum.
460 Reprod. 14, 997–1004.
461 North Atlantic Salmon Conservation Organization (NASCO) (2016). Report of the
462 twenty-second annual meeting of the Council of the North Atlantic Salmon Conservation
463 Organization.
464 Parodi, J., Ramírez-Reveco, A., Guerra, G., 2015. Example Use of low-cost system for
465 capturing the kinetic parameters of sperm cells in Atlantic salmon (*Salmo salar*). Adv.
466 Biosci. Biotechnol. 6, 63–72.
467 Pikitch, E.K., Doukakis, P., Lauck, L., Chakrabarty, P., Erickson, D.L., 2005. Status,
468 trends and management of sturgeon and paddlefish fisheries. Fish. Fish. (Oxf). 6(3), 233–
469 265.

Psenicka, M., Alavi, S.M.H., Rodina, M., Gela, D., Nebesarova, J., Linhart, O., 2007. Morphology and ultrastructure of Siberian sturgeon, *Acipenser baerii*, spermatozoa using scanning and transmission electron microscopy. Biol. Cell. 99, 103–115.

Ruban, G., Bin Zhu. 2010. *Acipenser baerii*. The IUCN Red List of Threatened Species 2010: e.T244A13046607.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture. 234, 1–28.

Sadeghi, S., Nuñez, J., Soler, C., Silvestre, M.A., 2017. Effect of the Activation Media with Different Osmolality and Cool Storage on Spermatozoa Motility Parameters over Time in Zebrafish, *Danio rerio*. Turkish J. Fish. Aquat. Sci. 17: 111–120.

Soler, C., Fuentes, M.C., Sancho, M., García, M., Núñez de Murga, M., Nuñez de Murga, J., 2012. Effect of counting chamber on seminal parameters, analyzing with the ISAS v1. Rev. Int. Androl. 10, 132–8.

Soler, C., Cooper, T., Valverde, A., Yániz, J., 2016. Afterword to Sperm morphometrics today and tomorrow special issue in Asian Journal of Andrology. Asian J. Androl. 18, 895–897.

Stephens, D.T., Hickman, R., Hoskins, D.D., 1988. Description, validation, and performance characteristics of a new computer-automated sperm motility analysis system. Biol. Reprod. 38, 577–586.

Stone, R., 2003. Freshwater eels are slip-sliding away. Science. 302, 221– 2.

Valverde, A., Madrigal, M., Caldeira, C., Bompart, D., Núñez de Murga, J., Arnau, S., Soler, C., 2018. Effect of frame rate capture frequency on sperm kinematic parameters and subpopulation structure definition in boars analysed with the ISAS®v1 CASA-Mot system. Reprod. Domest. Anim. *in press*.

494 Vantman, D., Koukoulis, G., Dennison, L., Zinaman, M., Sherins, R., 1988. Computer-
495 assisted semen analysis: Evaluation of method and assessment of the influence of sperm
496 concentration on linear velocity determination. Fertil. Steril. 49, 510–515.
497 Verstegen, J., Iguer-Ouada, M., Onclin, K., 2002. Computer-assisted semen analyzers in
498 andrology research and veterinary practice. Theriogenology. 57, 149–179.
499 Vladić, T.V., Afzelius, B.A., Bronnikov, G.E., 2002. Sperm Quality as Reflected Through
500 Morphology in Salmon Alternative Life Histories. Biol. Reprod. 66, 98–105.
501 Wilson-Leedy, J.G., Ingermann, R.L., 2007. Development of a novel CASA system based
502 on open source software for characterization of zebrafish sperm motility parameters.
503 Theriogenology. 67, 661–72.
504 Woolley, D.M., 1997. Studies on the eel sperm flagellum. I. The structure of the inner
505 dynein arm complex. J. Cell Sci. 110, 85–94.

3	α	SE_α	β	SE_β	MOT_α (%)	SE_{MOT}	MOT_{50} (%)	MOT_{100} (%)	MOT_{250} (%)
Eel									
x10									
10 µm	61.56	3.04	0.89	4.52	60.68	0.03	60.47	61.01	61.34
20 µm	56.25	3.76	2.47	5.87	53.83	0.09	53.54	54.88	55.70
x20									
10 µm	44.33	4.90	-3.34	9.72	47.80	0.23	47.39	45.84	44.93
20 µm	41.92	3.07	-11.23	6.05	54.80	0.63	52.48	46.90	43.85
Salmon									
x10									
10 µm	85.95	3.13	1.62	3.39	84.35	0.03	83.21	84.57	85.39
20 µm	86.16	2.45	0.86	2.54	85.30	0.01	84.69	85.42	85.86
x20									
10 µm	93.57	1.82	0.29	1.80	93.28	0.00	93.03	93.30	93.46
20 µm	82.08	5.6	1.84	6.35	80.26	0.05	79.11	80.58	81.48
Sturgeon									
x10									
10 µm	98.40	1.07	0.52	1.01	97.88	0.00	97.38	97.89	98.20
20 µm	96.84	1.80	0.52	1.73	96.32	0.01	95.84	96.34	96.64
x20									
10 µm	99.90	0.44	0.73	0.41	99.17	0.00	98.45	99.17	99.61
20 µm	96.77	1.30	-1.18	1.23	97.96	0.01	99.08	97.92	97.23

Table 1: Optimal FR needed to obtain the threshold level (α) for each technical condition, rate of increase the asymptote (β) and the asymptotic level of motility rate (MOT) for the sperm samples of the three diadromous fish species (eel, salmon, sturgeon). The theoretical MOT value for 50, 100 and 250 fps was calculated based on α and β values. Note: FR, frame rate; MOT, the percentage of total motility; α, threshold asymptotic level; β, the rate of increase; SE, standard error.

	α	SE_α	β	SE_β	VCL_α ($\mu m.s^{-1}$)	SE_{VCL}	VCL_{50} ($\mu m.s^{-1}$)	VCL_{100} ($\mu m.s^{-1}$)	VCL_{250} ($\mu m.s^{-1}$)
Eel									
x10									
10 µm	189.04	1.37	39.51	0.86	153.39	0.13	85.78	127.34	161.41
20 µm	203.08	1.66	46.11	0.96	161.83	0.17	80.75	128.06	168.88
x20									
10 µm	179.06	4.08	41.81	2.66	141.77	0.44	77.60	117.87	151.48
20 µm	188.88	3.31	40.16	2.09	152.70	0.33	84.60	126.41	160.85
Salmon									
x10									
10 µm	260.18	1.83	29.00	0.80	232.74	0.11	145.67	194.68	231.68
20 µm	253.36	2.00	27.88	0.85	226.96	0.11	145.07	191.72	226.62
x20									
10 µm	255.69	3.76	28.54	1.73	228.69	0.22	144.48	192.21	228.10
20 µm	253.08	3.99	29.39	1.85	225.33	0.24	140.60	188.63	225.01
Sturgeon									
x10									
10 µm	210.55	3.34	12.08	1.66	198.81	0.10	165.36	186.59	200.62
20 µm	227.70	2.62	17.77	1.22	210.61	0.11	159.59	190.63	212.08
x20									
10 µm	208.31	2.88	12.59	1.48	196.09	0.09	161.94	183.67	198.08
20 µm	227.93	4.81	15.94	2.26	212.53	0.18	165.71	194.35	213.85

Table 2: Optimal FR needed to obtain the threshold level (α) for each technical condition, rate of increase the asymptote (β) and the asymptotic level of VCL for the sperm samples of the three diadromous fish species (eel, salmon, sturgeon). The theoretical VCL value for 50, 100 and 250 fps was calculated based on α and β values. Note: FR, frame rate; VCL, curvilinear velocity; α, threshold asymptotic level; β, the rate of increase; SE, standard error.

	Eel	Salmon	Sturgeon
x10			
10 µm	60.73 ± 3.65^{x}	84.63 ± 3.37^{y}	98.27 ± 1.93
20 µm	55.06 ± 3.76	85.45 ± 43.37	96.20 ± 1.82
x20			
10 µm	46.87 ± 5.01^{y}	93.84 ± 4.80^{x}	99.76 ± 0.24
20 µm	49.39 ± 4.66	81.67 ± 4.80	100.00 ± 0.19

Table 3: Effect of the magnification lens and chamber at the optimal FR (100 fps) on the percentage of total motility for the sperm samples of European eel, Atlantic salmon and Siberian sturgeon. Data are presented as mean ± SEM. Last letters of the alphabet indicate a significant difference between the magnification lens within the same chamber ($P < 0.05$). Note: x10, x10 objective; x20, x20 objective; 10 µm, 10 µm depth; 20 µm, 20 µm depth.

	VCL ($\mu m\ s^{-1}$)	VSL ($\mu m\ s^{-1}$)	VAP ($\mu m\ s^{-1}$)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Eel								
x10								
10 µm	$155.70 \pm 1.25^{b,x}$	44.10 ± 0.69^{b}	95.36 ± 0.94^{x}	25.38 ± 0.28	$41.83 \pm 0.36^{b,y}$	$58.27 \pm 0.27^{a,x}$	$1.17 \pm 0.01^{b,x}$	31.21 ± 0.33^{y}
20 µm	$164.31 \pm 1.31^{a,x}$	46.16 ± 0.73^{a}	97.61 ± 0.99^{x}	25.78 ± 0.29^{y}	$42.87 \pm 0.37^{a,y}$	57.48 ± 0.28^{b}	$1.21 \pm 0.01^{a,x}$	30.84 ± 0.35^{y}
x20								
10 µm	144.61 ± 4.17^{y}	40.51 ± 2.18	$82.72 \pm 2.92^{b,y}$	26.93 ± 0.89	45.10 ± 1.13^{x}	55.38 ± 0.79^{y}	0.97 ± 0.02^{y}	41.05 ± 1.21^{x}
20 µm	153.42 ± 2.70^{y}	44.44 ± 1.42	$90.27 \pm 1.89^{a,y}$	27.04 ± 0.57^{x}	45.97 ± 0.74^{x}	56.96 ± 0.51	1.01 ± 0.02^{y}	41.63 ± 0.78^{x}
Salmon								
x10								
10 µm	238.19 ± 1.92^{x}	$118.13 \pm 1.90^{b,y}$	169.60 ± 1.46^{x}	$48.90 \pm 0.67^{b,y}$	$68.26 \pm 0.85^{b,y}$	71.10 ± 0.31^{x}	$1.39 \pm 0.01^{a,x}$	74.45 ± 0.89^{y}
20 µm	236.48 ± 2.05	126.19 ± 2.02^{a}	167.79 ± 1.56^{x}	52.64 ± 0.71^{a}	74.10 ± 0.91^{a}	70.67 ± 0.33^{x}	$1.36 \pm 0.01^{b,x}$	73.48 ± 0.95^{y}
x20								
10 µm	228.10 ± 3.48^{y}	131.77 ± 2.98^{x}	156.81 ± 2.35^{y}	$57.59 \pm 1.05^{a,x}$	$82.82 \pm 1.33^{a,x}$	68.78 ± 0.53^{y}	1.16 ± 0.02^{y}	100.48 ± 1.50^{x}
20 µm	229.03 ± 4.05	124.51 ± 3.46	157.23 ± 2.73^{y}	53.99 ± 1.22^{b}	77.51 ± 1.54^{b}	69.06 ± 0.61^{y}	1.18 ± 0.02^{y}	99.74 ± 1.75^{x}
Sturgeon								
x10								
10 µm	202.58 ± 4.68	141.06 ± 4.51	177.92 ± 4.10	68.29 ± 1.67	77.62 ± 1.63^{y}	$87.01 \pm 0.94^{a,x}$	1.14 ± 0.02^{x}	53.17 ± 1.67^{y}
20 µm	208.68 ± 2.95	138.76 ± 2.85^{y}	175.79 ± 2.59	66.13 ± 1.05	78.20 ± 1.03^{y}	84.05 ± 0.59^{b}	1.19 ± 0.01^{x}	53.74 ± 1.06^{y}
x20								
10 µm	198.00 ± 3.45^{b}	143.38 ± 3.92	169.17 ± 2.95^{b}	71.05 ± 1.39	82.47 ± 1.37^{x}	$84.77 \pm 0.64^{a,y}$	$0.90 \pm 0.02^{b,y}$	67.90 ± 1.17^{x}
20 µm	217.95 ± 4.47^{a}	152.45 ± 5.08^{x}	178.88 ± 3.82^{a}	68.40 ± 1.80	82.00 ± 1.77^{x}	82.07 ± 0.83^{b}	$1.04 \pm 0.02^{a,y}$	68.53 ± 1.52^{x}

Table 4: Effect of the magnification lens and chamber at the optimal FR on estimated kinematic parameters of European eel (200 frames s^{-1}), Atlantic salmon (250 frames s^{-1}) and Siberian sturgeon (225 frames s^{-1}). First letters of the alphabet indicate significant differences between chamber within the same magnification lens ($P < 0.05$); last letters of the alphabet indicate a significant difference between magnification lens within the same chamber ($P < 0.05$). Note: VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; x10, x10 objective; x20, x20 objective; 10 µm, 10 µm depth; 20 µm, 20 µm depth.

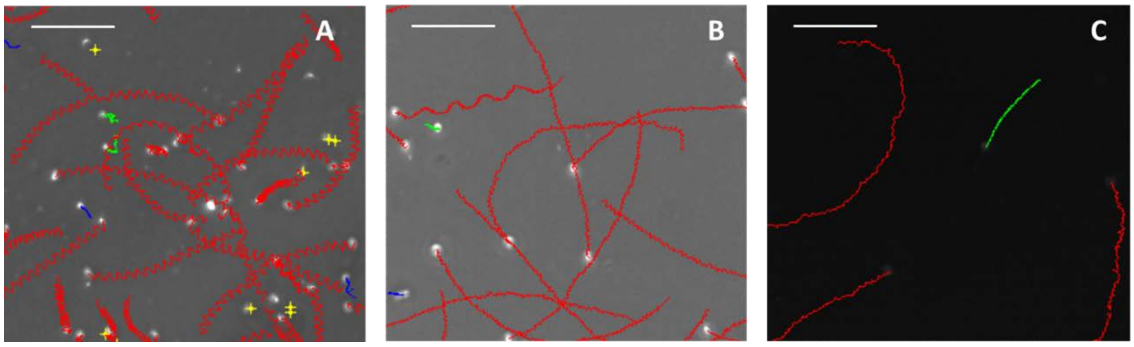

Figure 1: Sperm motility tracks of (A) European eel, (B) Atlantic salmon and (C) Siberian sturgeon, exhibiting 4 groups of spermatozoa velocity: rapid (red), medium (green), slow (blue) and static (yellow). Scale bar of 10 µm.

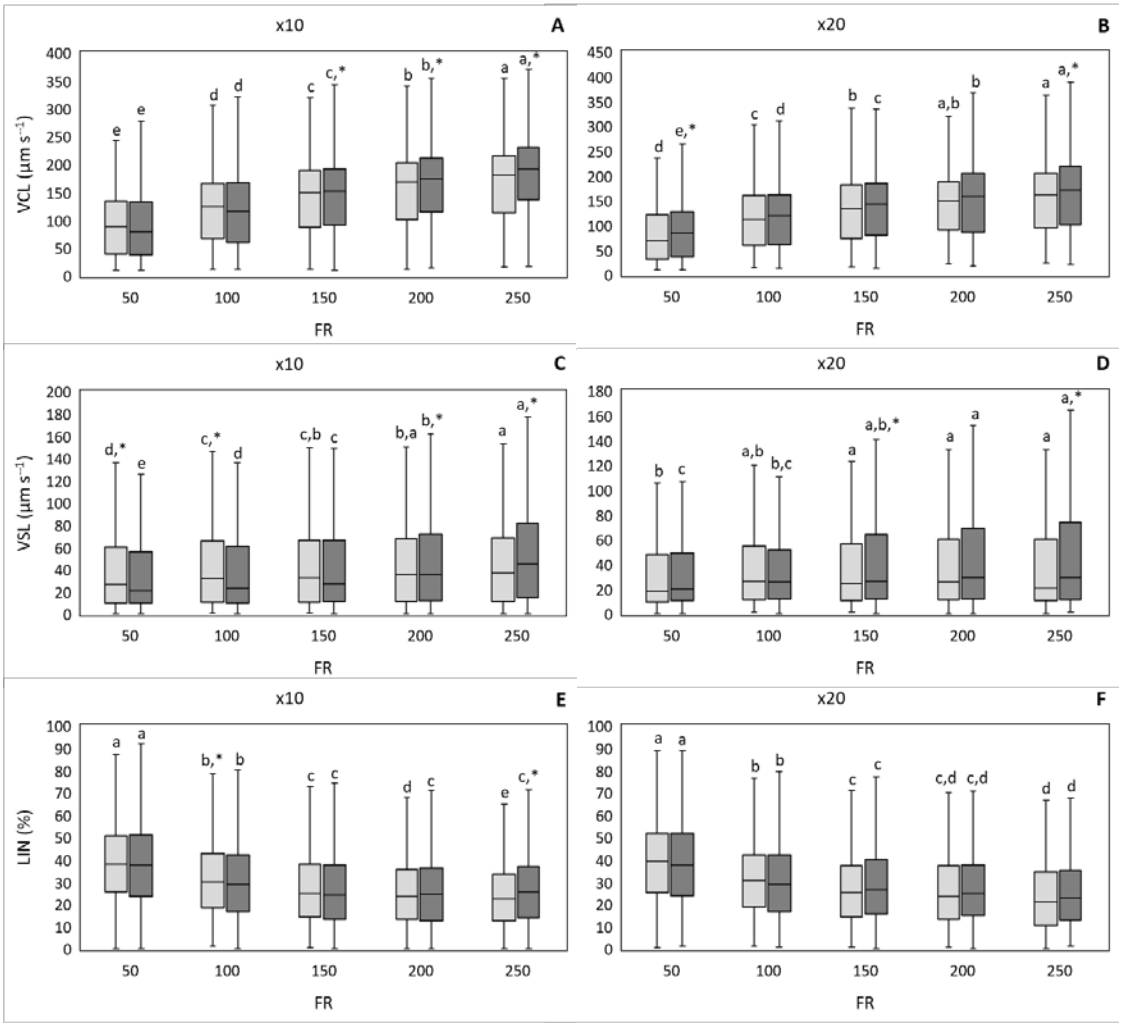

Figure 2: Effect of magnification lens (x10 and 20x), FR (up to 250 fps) and chamber (10 (light grey boxplot) and 20 µm (dark grey boxplot) depth) on VCL (A, B), VSL (C, D) and LIN (E, F) of European eel sperm. Data are presented as median (interquartile range; Q1 and Q3) and minimum and maximum values. Different letters indicate significant differences between FR within the same magnification lens and chamber ($P < 0.05$); the asterisk (*) indicate a significant difference between chamber within the same magnification lens and FR ($P < 0.05$).

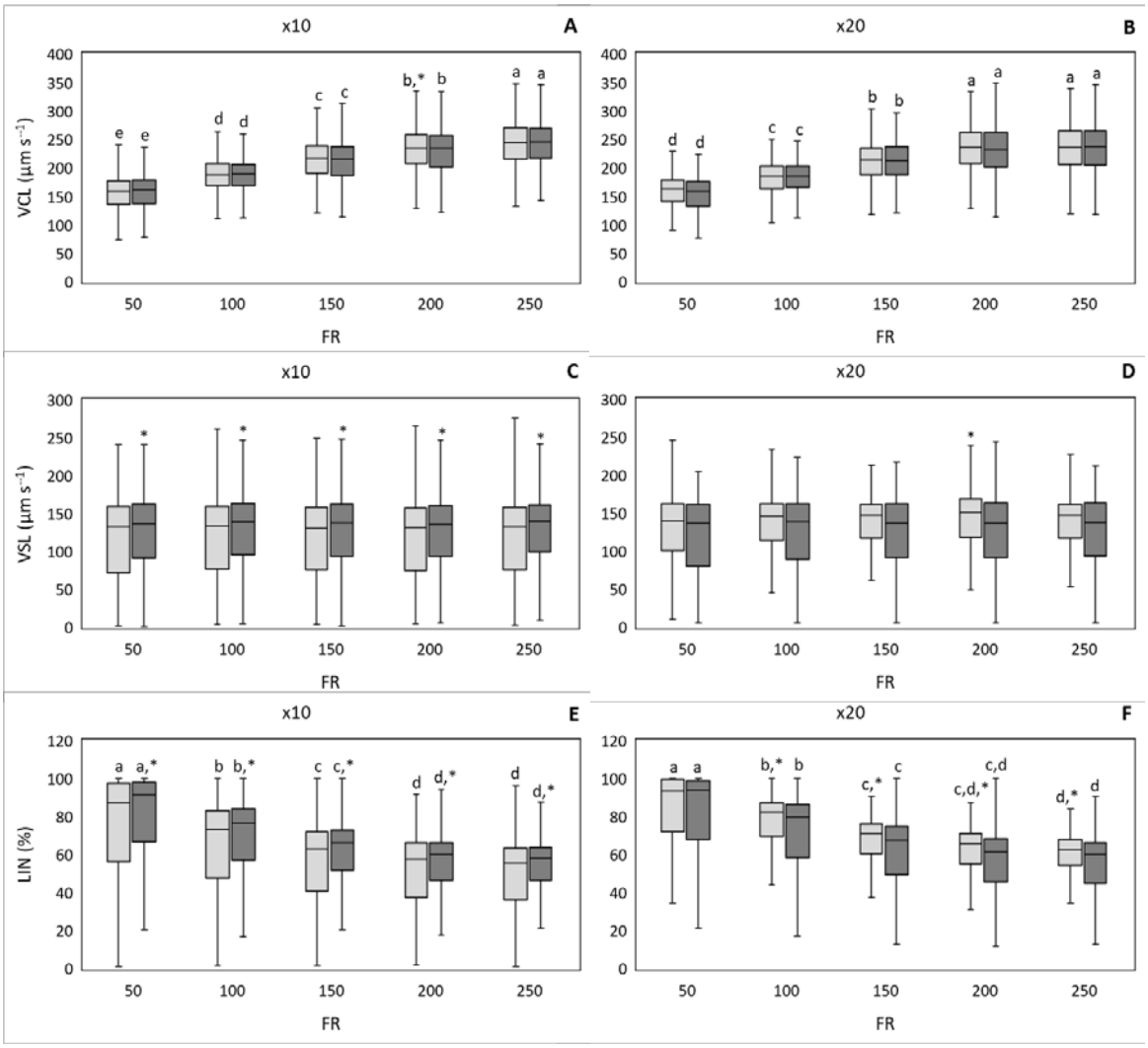

Figure 3: Effect of magnification lens (x10 and 20x), FR (up to 250 fps) and chamber (10 (light grey boxplot) and 20 µm (dark grey boxplot) depth) on VCL (A, B), VSL (C, D) and LIN (E, F) of Atlantic salmon sperm. Data are presented as median (interquartile range; Q1 and Q3) and minimum and maximum value. Different letters indicate significant differences between FR within the same magnification lens and chamber ($P < 0.05$); the asterisk (*) indicate a significant difference between chamber within the same magnification lens and FR ($P < 0.05$).

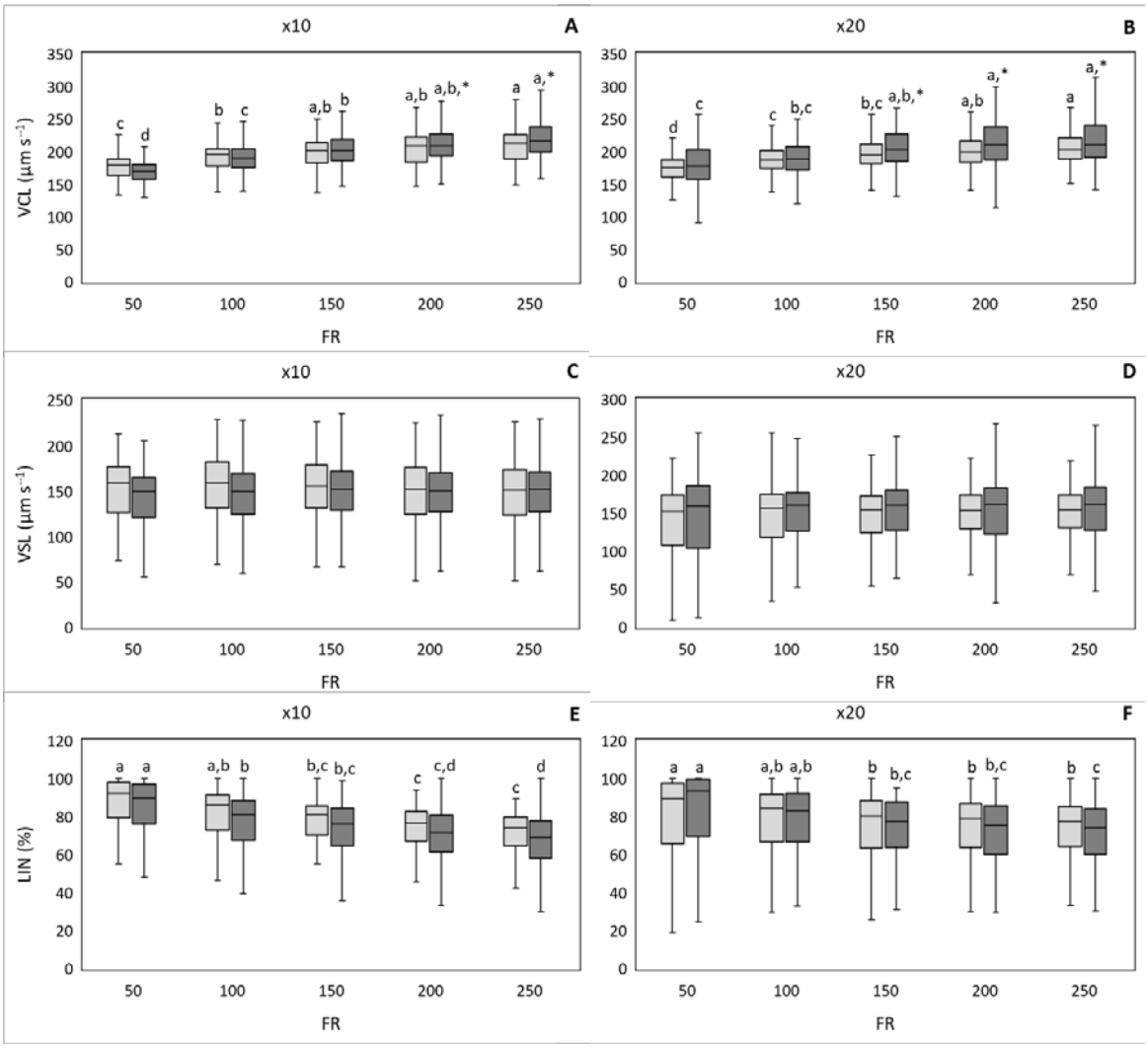

Figure 4: Effect of magnification lens (x10 and 20x), FR (up to 250 fps) and chamber (10 (light grey boxplot) and 20 µm (dark grey boxplot) depth) on VCL (A, B), VSL (C, D) and LIN (E, F) of Siberian sturgeon sperm. Data are presented as median (interquartile range; Q1 and Q3) and minimum and maximum value. Different letters indicate significant differences between FR within the same magnification lens and chamber ($P < 0.05$); the asterisk (*) indicate a significant difference between chamber within the same magnification lens and FR ($P < 0.05$).