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Subjective and objective assessment of fish sperm motility: when the technique and technicians matter

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Abstract

Fish sperm motility is nowadays considered the best sperm quality biomarker in fish, and can be evaluated both by subjective and computerized methods. With the aim to compare the precision and accuracy of both techniques, fish sperm samples were assessed by subjective methods and by a computer assisted sperm analysis (CASA-Mot) system, and simultaneously by three different technicians with different degree of expertise on the sperm quality analysis. Statistical dispersion parameters (CV, coefficient of variation; and RG, range) were estimated in order to determine the precision and accuracy of the techniques and the influence of laboratory staff on sperm motion assessments.

Concerning precision, there were not much significant differences between the technical support staff (high, medium, and low experimented technician), and statistical dispersion parameters were quite similar between them independently of the technique used and the sperm motility class analyzed. However, concerning accuracy, experimented technician reported subjective motility values very closed to the values provided by the CASA-Mot system, only 10 percentage points away from the data provided by a CASA-Mot system. However, medium and low-experimented technicians often overestimate the CASA-Mot values, and amplitudes up to 30 percentage points were detected in several sperm assessments.

To sum up, both the technique (subjective or objective) and the technician (degree of expertise) became key factors in order to reach accurate motility estimations, so the use of both qualified staff and novel CASA-Mot systems seem to be a critical requirement for obtaining satisfying results in fish species with similar motility patterns.

Keywords:

Eel; spermatozoa; CASA-Mot; velocity; kinetic; accuracy; precision

1. Introduction

Over the years, a relatively high number of sperm parameters have been used to assess sperm quality in fish (Fauvel et al. 2010). These sperm biomarkers have so far been documented in scientific articles, and several traits such as osmolality, plasma composition, sperm density or sperm morphology have been linked to the ability of sperm to fertilize the ova (reviewed by Cabrita et al. 2014). However, sperm motility is currently considered the most useful tool for assessing sperm quality in fish, and high correlations have been reported between sperm motility and fertilization or hatching rates in several fish species such as pufferfish (*Takifugu niphobles*; (Gallego et al. 2013b)), rainbow trout (*Oncorhynchus mykiss*; Bozkurt and Secer 2006), red seabream (*Pagrus major*; Liu et al. 2007) or tambaqui (*Colossoma macropomum;* Gallego et al. 2017).

Nowadays, sperm motility evaluation can be done by two different ways in the laboratory: *i*) the subjective way, in which a technician (more or less experienced), make an evaluation of sperm motility through a simple observation under the microscope; and *ii)* the objective way, in which sperm analysis systems, particularly CASA-Mot (Computer Assisted Sperm Analysis) system, integrates the successive positions of the heads of moving spermatozoa in every frame video-taped for calculating their trajectories and kinetic characteristics.

Subjective evaluation method has been the most used technique to evaluate sperm motility over the history, but some problems have emerged from this method (Rurangwa et al. 2004). First drawback is focused on the own limitation of human eye, through which we can only provide a coarse evaluation of *i*) the percentage of motile spermatozoa and ii) the sperm motility duration. In addition, this type of evaluation depends on the observer's experience, and several aspects such as sperm density, sperm velocity, drift, etc. can cause over- or underestimations (Hala et al. 2009). Therefore, the low reproducibility of this subjective assessment, which can result in variations of 30 to 60% of CV (coefficient of variation) from the same sample, often makes difficult to interpret and compare the results intra- and inter-labs (Verstegen et al. 2002; Rosenthal et al. 2010). By contrast, the gradual appearance and popularization of CASA-Mot systems has made possible to estimate a higher number of sperm motion parameters not given by subjective evaluation (spermatozoa velocities, motion pattern models, sperm subpopulations, etc.), and do it in an objective, sensitive and accurate way (Kime et al. 2001). Nevertheless, it is important to consider that CASA-Mot systems are not ready-to-use devices, and they

also depend largely on technical and biological settings which need to be standardized for enhancing the comparability of data produced by different research groups (Boryshpolets et al. 2013; Gallego et al. 2013a). In addition, CASA-Mot systems are not available for many research groups due to the initial investment necessary to purchase the complete equipment (software, high-resolution camera, etc.), so half of the scientific studies carried out during the last years have not used a CASA-Mot systems for the spermatozoa motion assessment (Gallego and Asturiano 2018).

In this scenario, technique and technicians could have an important role for obtaining credible assessments of spermatozoa kinetic features, so the aim of this study was to compare the precision and accuracy of both subjective and objective techniques and, simultaneously, the influence of laboratory staff previous experience on sperm motion assessments.

2. Material and Methods

2.1 Fish handling and sperm collection

Thirty adult European eel males from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Spain) were moved to the Aquaculture Laboratory of the Universitat Politècnica de València (Spain). The fish were distributed in two 150-L aquaria (approximately 15 males per aquarium) keeping a constant temperature of 20 ºC and covered to reduce light intensity and fish stress. During one week, the eels were gradually acclimatized from freshwater to sea water (salinity = 37 ± 0.3 g/l). Later they were anaesthetized once a week with benzocaine (60 ppm) for injecting 1.5 IU g^{-1} fish of recombinant human chorionic gonadotropin (Ovitrelle, Merck S.L., Madrid). Fish were fasted throughout the trial and they were handled in accordance with the European Union regulations regarding the protection of experimental animals (Dir 86/609/EEC).

From the 7^{th} week of hormonal treatment, sperm samples were weekly collected by abdominal pressure 24 h after the administration of the hormone (following the protocol described by Pérez et al. 2000), and taking special care to avoid the contamination with faeces, urine and seawater. Samples were diluted 1:9 (sperm:extender) in P1 medium (Peñaranda et al. 2010) and kept in plastic tubes at 4 ºC until sperm kinetic analyses, which were carried out during the next 2 hours after sperm collection.

2.2 Experimental design

Each of the samples was evaluated according the Figure 1 by three different techniques: *i*) by subjective way (human eye) directly through the ocular lens (eyepieces) of the microscope, *ii*) by subjective way (human eye) using a computer monitor connected to the microscope, and *iii*) by an objective way using a CASA-Mot system. The main difference between the 2 subjective assessments was that sperm sample observed directly through the eyepieces was done in a bright-field microscopy (dark cells on bright background) with a great wide field of view; while the assessment through the screen (monitor) was done in a dark-field (bright cells on dark background) with a smaller wide field of view. In addition, these three assessing methods were carried out by three different technicians with different degree of expertise on the sperm quality analysis: *i*) a high experimented technician (High ET; a postdoctoral researcher) with years of experience on sperm motility assessment, *ii*) a medium experimented technician (Medium ET; a pre-doctoral student) whose thesis is focused on issues related to sperm motion analysis, and finally *iii*) a low experimented technician (Low ET; a Grade student) with very little experience on the sperm quality analysis. It is important to remark that the dispersion parameters (see section 2.5) used in this study were estimated analyzing the same sample through three consecutive sperm activations for each technique.

2.3 Sperm motility assessment both by subjective and objective methods.

Each sample was activated by mixing 0.5 µl of P1-diluted sperm (see section 2.1) with 4.5 µl of artificial sea water (Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH was adjusted to 8.2). All the motility analyses (both by subjective or objective methods) were performed by triplicate.

In relation to the subjective method, technicians estimated the sperm motility (percentage of motile spermatozoa) by both *i*) looking directly through the eyepieces of the microscope and *ii*) looking directly through computer monitor. Spermatozoa were considered motile presenting any type of movement (progressive or non-progressive according the World Health Organization (WHO) criteria in the 5th edition).

In addition, technicians were asked to classify every sample as Fast (spermatozoa with fast progressive movement), Medium (spermatozoa with medium forward movement), or Slow (spermatozoa with slow forward movement or non-progressive movement) depending on the motion (estimated subjectively) of swimming spermatozoa. Finally, objective assessments were done immediately after subjective evaluation using a CASA-

Mot system, and several kinetic parameters such as total motility (MOT, %), progressive motility (pMOT, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and average path velocity (VAP, µm/s) were recorded for further analysis. Several manuscripts have reported high correlations between these parameters with fertilization and hatching rates in several fish species, so they become good biomarkers to predict and sperm quality and carrying out sperm studies (Gallego and Asturiano, 2018).

In order to perform an in-depth analysis of the results, sperm samples were classified into three classes based on the percentage of motile spermatozoa provided by the CASA-Mot system: Class I (C-I) = 0-25% of motile cells; Class II (C-II) = 26-50% of motile cells; and Class III (C-III) = 51-100% of motile cells.

2.4 Setting used on CASA-Mot system.

Kinetic sperm analysis were carried out by the motility module of ISAS®v1 (Proiser R+D, S.L.; Paterna, Spain) using an ISAS® 782M camera recorder capturing 60 frames per second (fps). Between 200 and 600 spermatozoa were captured in each field adjusting the brightness and contrast in the CASA-Mot settings in relation to the microscope light with the aim to reach spermatozoa clearly defined (Gallego et al. 2013a). Range size particle were defined between 2 and 20 µm and spermatozoa were considered immotile if their VCL was lower than 10 µm/s.

2.5 Statistical analysis

For evaluating the variability on the dataset, several measures of dispersion such as the coefficient of variation (CV, %) and the absolute range (RG, difference between the smallest value and the largest value of a series) were estimated both for each method and for each technician (observer).

In order to evaluate the accuracy, the amplitude (difference between the subjective evaluation and the motility values provided by a CASA-Mot system) were estimated. Coefficients of correlation (*r*) between the subjective and objective assessments were also obtained for High, Medium, and Low experimented technicians (ET) among different sperm motility classes (C-I, C-II and C-III). Finally, box plots were created in order to assess the ability of each technician to appreciate the velocity of swimming spermatozoa. Data expressed in percentages were transformed using the arcsine transformation, and Shapiro-Wilk test was used to check the normality of data distribution. One-way analysis of variance (ANOVA) was used to analyse the data and significant differences between

189 treatments were detected using the Tukey multiple range test ($P<0.05$). Statistical
190 analyses were performed using the statistical package SPSS version 19.0 for Windows
191 software (SPSS Inc., Chicago, IL, USA).
192
193 # 3. Results
194 ## 3.1. Precision of techniques & technicians
195 The precision for both techniques and technicians was evaluated through CVs and RG
196 values (see Figure 2 and 3, respectively). CVs were quite similar between technicians
197 independently of the technique used and the sperm motility class analyzed (Fig. 2), and
198 statistical differences were only found assessing samples from C-II and C-III through a
199 subjective motility analysis (Fig. 2A and 2B).
200 Regarding the absolute range (RG, defined as the difference between the smallest value
201 and the largest value registered in the same motility assessment), a similar pattern than
202 obtained in CVs were found. However, trends in RG showed that high ET showed smaller
203 RGs than medium and low ETs independently of the technique applied and the sperm
204 motility class analyzed (Fig. 3). Nevertheless, statistical differences were only found
205 assessing samples from C-II and C-III through a subjective motility analysis (Fig. 3A and
206 3B).
207
208 ## 3.2. Accuracy of techniques & technicians
209 The ability of technicians to carry out an accurate subjective evaluation was measured as
210 the difference (amplitude) between the CASA-Mot motility values and the subjective
211 estimations (Figures 4 and 5). Concerning subjective motility assessments carried out
212 through the eyepieces of the microscope, high ET obtained subjective motility values
213 relatively closed to CASA-Mot motility values, presenting over or under estimations of
214 only around 10 percentage points throughout all the sperm motility classes (Fig. 4A).
215 However, although medium and low ETs had acceptable amplitude values in C-I class,
216 overestimation of values was the common trend in samples belonging to C-II and C-II
217 classes, with subjective sperm motility values 25 percentage points higher than the
218 motility assessed by a CASA-Mot system (Figs. 4B and 4C).
219 Concerning subjective motility values obtained through the computer monitor (screen),
220 high ET also obtained subjective motility values relatively closed to real motility values
221 assessed by a CASA-Mot system, presenting once again over or under estimations of

around 10% along all the sperm motility classes (Fig. 5A). Medium ET was able to estimate good subjective values (relatively closed to CASA-Mot motility values) of the samples belonging to C-I and C-II classes, but underestimations (up to 16%) were the common pattern on the C-III class (Fig. 5B). Finally, low ET was not able for estimating subjective motility values closed to CASA-Mot assessments, and high overestimations were the common trend in all the sperm classes, reaching amplitude values up to 25 and 31% in C-I and C-II classes, respectively (Fig. 5C).

Coefficients of correlation provided in Table 1 show that although all technicians showed relatively high *r*-values among C-I and C-III classes (>0.8 and >0.7, respectively), High ET was the only technician able to reach acceptable *r*-values in samples belonging to C-II class. In this sense, Medium and Low ETs presented low *r*-values (0.42 and 0.57, respectively) between the subjective microscope assessments and CASA-Mot estimations.

3.3. Technician ability for estimating sperm velocities

Finally, last trial tried to evaluate the technician ability for estimating sperm velocities using the subjective assessments. In relation to subjective estimations carried out through the eyepieces of the microscope (Fig. 6), spermatozoa classified as Fast, Medium or Slow by the high ET showed significant differences both in terms of VCL, VSL and VAP. However, spermatozoa classification carried out by medium and low ET did not reveal statistical differences between slow and medium spermatozoa in terms of VSL and VAP, evidencing their incapacity to evaluate properly the spermatozoa velocity.

Concerning subjective estimations carried out through the computer monitor (Fig. 7), spermatozoa classified as Fast, Medium or Slow both by the high and medium ET showed significant differences in terms of VCL, VSL and VAP, so both observers were able to do an accurate estimation of sperm velocity. However, velocities of spermatozoa classified as slow and medium by low ET did not differ statistically neither in VCL, VSL and VAP, so low ET was only able to distinguish subjectively the fast spermatozoa to the rest.

4. Discussion

This study show, by the first time in fish species, the importance of technique and technicians chosen for obtaining credible sperm motility assessments to be applied in fish

spermatology research. Both precision and accuracy parameters were obtained in order to investigate the effect of subjective or objective methods for assessing sperm motility, at the same time that ability of different technicians (with different degree of experience) for carrying out the different analysis.

In relation to precision, which reflects how consistent results are when measurements are repeated (even if they are far from the "real" value), the data revealed that there were not much differences depending on the methods used (objective or subjective), and CVs were quite similar between the techniques applied. In this sense, CVs are often used for testing analytical or instrumental techniques (immunoassay tests, PCR plates, etc…), and values no bigger than 25% are usually accepted in the scientific field (McAuliffe et al. 2015). Even though there are not data from fish, CV values obtained from subjective and objective assessment techniques were similar than reported in several mammal species. For example, in rams, CVs of sperm motility assessments ranged between 12.5 to 31.74% (Komatireddy and Madishetti 2017); on boar, CVs values ranged from 4.7 to 34.7% (Reicks et al. 2012); and in bull, CVs ranged between 21 to 44% (Pepper-Yowell 2011). On the other hand, there were not much significant differences between the technical support staff (high, medium, and low experimented technicians), and statistical dispersion parameters were quite similar between them independently of the technique used and the sperm motility class analyzed. In this respect, the degree of experience in the laboratory did not become a key factor in order to achieve a high level of precision in fish sperm motility assessments.

However, in relation to accuracy, which measure the ability of technicians to carry out an accurate subjective evaluation by the difference (amplitude) between the CASA-Mot motility values and the own subjective assessment, this study yielded interesting results. When sperm motility assessments were carried out through the eyepieces of the microscope, high ET obtained subjective motility values relatively closed to the values assessed by a CASA-Mot system (with over- or under-estimations of only around 10%), However, medium and low ETs provided overestimated values up to 25 percentage points, so the data reveal that the degree of experience in the laboratory become a key factor in order to achieve a high degree of accuracy (even though sometimes the low ET obtained more accurate results than the medium ET).

On their hand, when subjective motility values were obtained through the computer monitor (screen), both high and medium ET were able to improve their assessment performance, and subjective values provided for them were closer to the CASA-Mot

values. These results can be explained thanks to image quality field because while the sperm samples are analyzed directly by the microscope, spermatozoa trajectories are difficult to distinguish in the clear field, and the overlap of trajectories can cause erroneous assessments of the samples; however, when sperm motion is assessed subjectively by the computer monitor (screen), spermatozoa appear clear over the dark field to the observer (technician), then accurate assessments can be carried out. In this sense, coefficients of correlation support this hypothesis, and both High, Medium and Low ETs presented higher *r*-values (r=0.78-0.96) in assessments carried out by the computer monitor (screen) instead of the rude microscope evaluation (r=0.42-0.92). Therefore, when sperm motility assessment is carried out without CASA-Mot system, it is recommended to assess the motility by the computer monitor (screen) instead of directly by the eyepieces of the microscope.

On the other hand, it is important to note that *r*-values obtained for samples belonging to CII (r=0.42-0.65) were much lower than obtained for CI- and C-III classes (r=0.71-0.92), overall for the medium and low ETs. These results show that samples with motilities between 25 and 50% have more difficulties for their accurate analyses, so subjective results can be compromised when the sperm samples are analysed in this range of motility. Similar results have been reported in other species in which, although technicians were able to differentiate correctly the extremes of the sperm motility scale, the samples ranging between 34 to 57% were highly divergent for different technicians (Walker et al. 1982). In fact, the subjective evaluation in Walker's study was not capable of defining this boundary (limit), and fertility workups on males are incorrect 14 times out of 15 in this critical range, so the use of CASA-Mot systems seem to be an essential tool for working in fertility trials.

In relation to technician ability for estimating sperm velocities by subjective assessments, high experimented technician was able to distinguish fast, medium and low spermatozoa, while less experimented technicians were not able to do it, evidencing their incapacity to evaluate properly the spermatozoa velocity. On this regard, sperm velocities seems to be the major component that determines fertilization success and the proportion of the paternity through the sperm competition in several fish species (Gage et al. 2004; Rudolfsen et al. 2008; Gasparini et al. 2010), so technician ability for predicting velocity classes can be a useful tool to carry out fertilization trials in the aquaculture sector, optimizing the reproductive efficiency in the fish farms (Gallego et al. 2013b). The data obtained in this study suggest that the degree of expertise of a technician on the sperm

quality analysis seems to be a key factor to predict velocities, and even though having a CASA system to make accurate assessments is the most recommended option, high experimented technicians are a requirement for investigating male fertility status as well as monitoring spermatogenesis.

To sum up, this study showed, by the first time in fish species, the importance of technique and technicians chosen for obtaining credible sperm motility assessments to be applied in fish spermatology research. Both the technique (subjective or objective) and the technician (degree of expertise) became key factors in order to reach accurate motility estimations, so the use of both qualified staff and novel CASA-Mot systems seem to be a critical request for obtaining satisfying results in species that have a motility pattern similar to that of the European eel.

In addition, because there are many different configurations and methods of using CASA-Mot systems, it is important to establish standard methods of enhancing the reliability, comparability, and applicability of data produced by different research groups (Castellini et al. 2011; Boryshpolets et al. 2013; Gallego et al. 2013a). All studies that use CASA must describe its methodology very clearly, particularly concerning image acquisition rate, track sampling time, number of cells sampled, type and depth of the chamber used, microscope magnification, etc. in order to make it possible to compare the results obtained by different laboratories,

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Figure legends

Figure 1. Experimental design for carrying out the motility assessments through the three different techniques (Microscope, Screen, and CASA-Mot system) and three techicians with different degree of experience (High, Medium, and Low). Each sperm sample was assessed consecutively by the three methods and the same observer in order to avoid differences between the different evaluation methods. Samples were evaluated in different order with every technique to avoid the observer´s preconception on the grade of motility of the sample from the technique used previously.

Figure 2. Coefficients of variation (CVs) obtained by High, Medium, and Low experimented technicians (ETs) among different sperm motility classes (C-I, C-II and C-III). Sperm motility was assessed through (A) the eyepieces of the microscope, (B) the computer monitor (screen), or (C) by a CASA-Mot system. Different letters indicate statistical differences ($P \leq 0.05$) between different technicians.

Figure 3. Absolute ranges (RGs, difference between the smallest value and the largest value of a series) obtained by High, Medium, and Low experimented technicians (ET) among different sperm motility classes (C-I, C-II and C-III). Sperm motility was assessed through (A) the eyepieces of the microscope, (B) the computer monitor or screen, or (C) by a CASA-Mot system. Different letters indicate statistical differences ($P \leq 0.05$) between different technicians.

Figure 4. Differences (amplitude) between the sperm motility values provided by a CASA-Mot system and the sperm motility assessments carried out through the eyepiece of the microscope by a High (A), Medium (B), and Low (C) experimented technicians (ETs).

Figure 5. Differences (amplitude) between the sperm motility values provided by a CASA-Mot system and the sperm motility assessments carried out through the computer monitor by a High (A), Medium (B), and Low (C) experimented technicians (ETs).

Figure 6. Velocity values (VCL, VSL and VAP) provided by a CASA-Mot system from samples classified by different technicians as Fast (FA), Medium (ME), or Slow (SL).

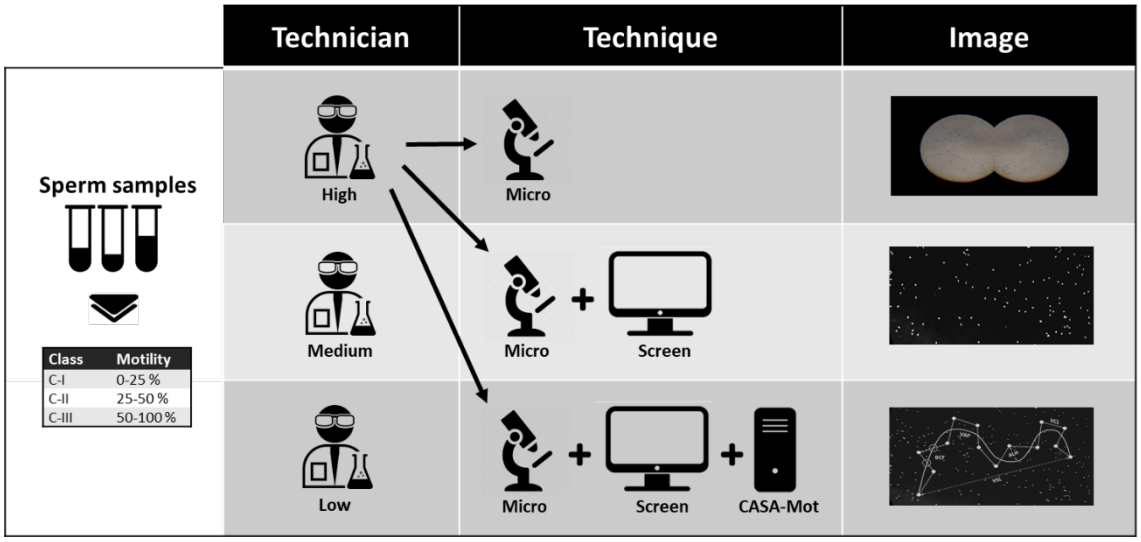
Velocity estimations (FA, ME, and SL) provided by High, Medium, and Low experimented technicians (ETs) were carried out through the eyepiece of the microscope. Different letters indicate statistical differences ($P \leq 0.05$) between sperm velocity classes.

Figure 7. Average velocity values (VCL, VSL and VAP) of spermatozoa classified by different technicians as Fast (FA), Medium (ME), or Slow (SL). Velocity estimations (FA, ME, and SL) provided by High, Medium, and Low experimented technicians (ETs) were carried out through the computer monitor (screen). Different letters indicate statistical differences ($P \leq 0.05$) between sperm velocity classes.

Table legends

Table 1. Coefficients of correlation (*r*) between the sperm motility values assessed subjectively by eyepieces of the microscope (micro) and through the computer monitor (screen) with the sperm motility values provided by a CASA-Mot system. *r* were estimated for High, Medium, and Low experimented technicians (ET) among different sperm motility classes (C-I, C-II and C-III).

Figure 1


Technician
Technique
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Sperm samples
Class
Motility
C-I
0-25 %
C-II
25-50 %
C-III
50-100 %
High
Micro
Medium
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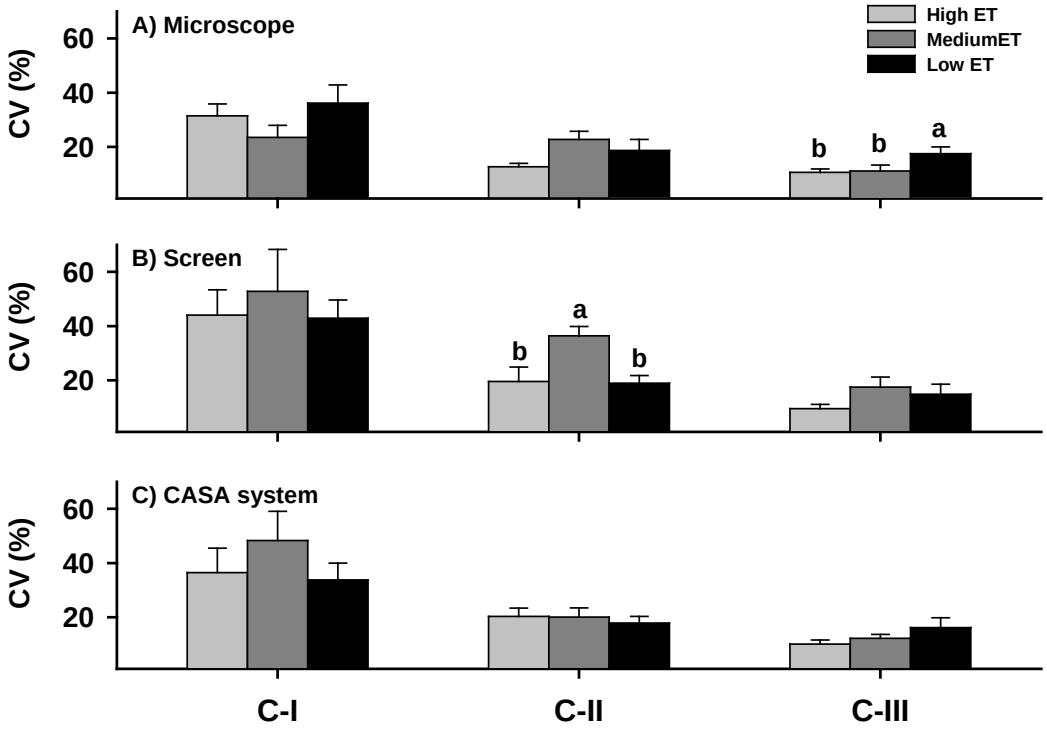
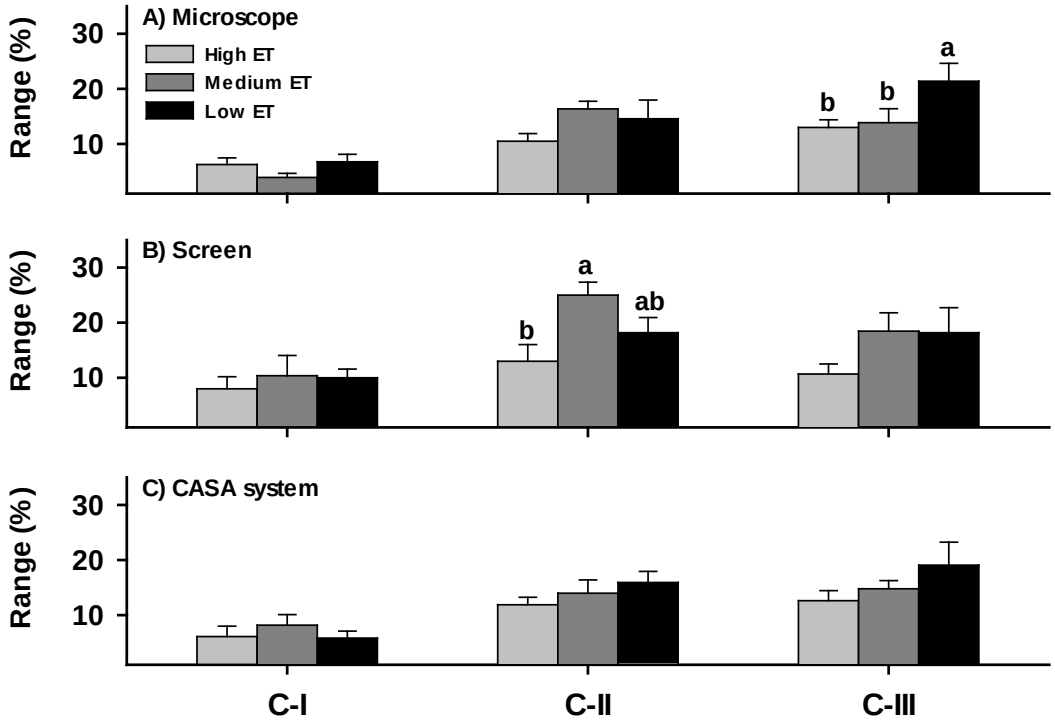
Screen
Low
Micro
Screen
CASA-Mot

475 **Figure 2**



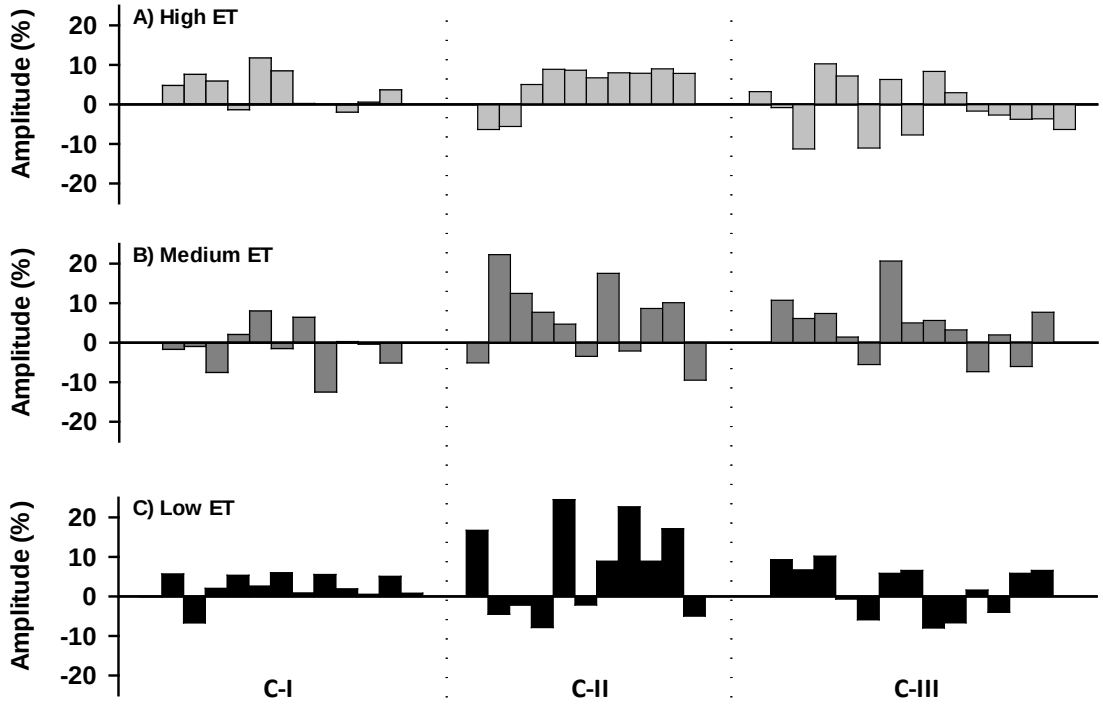
476

477 **Figure 3**



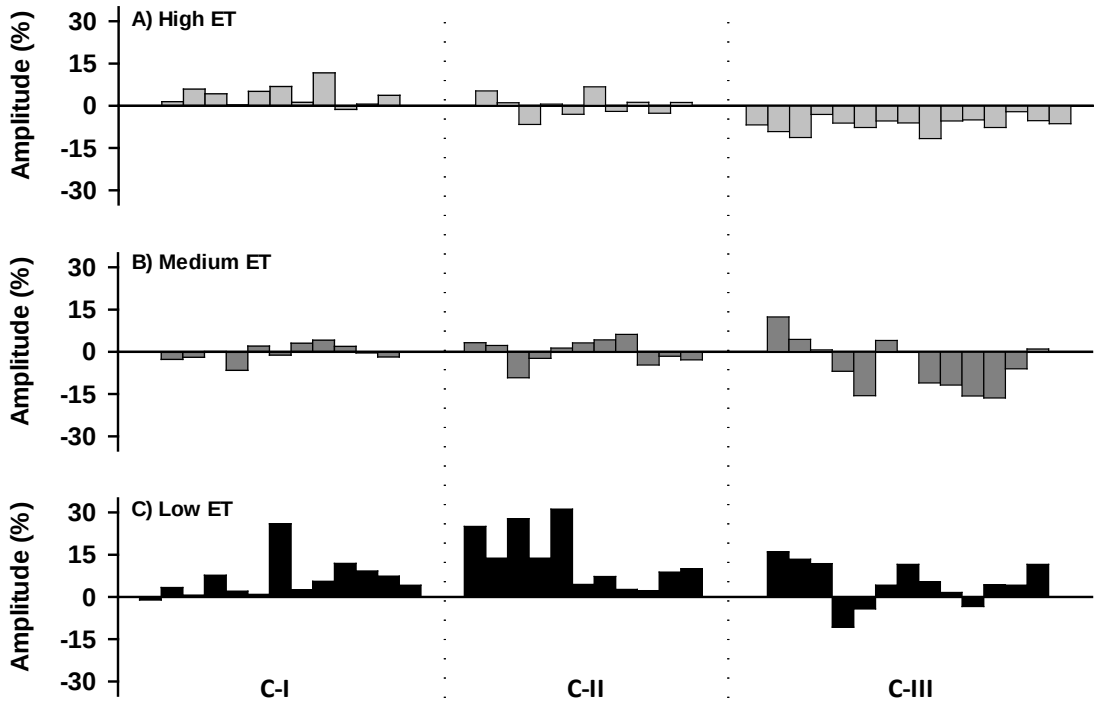
478

479 **Figure 4**



480

481 **Figure 5**



482

Figure 6

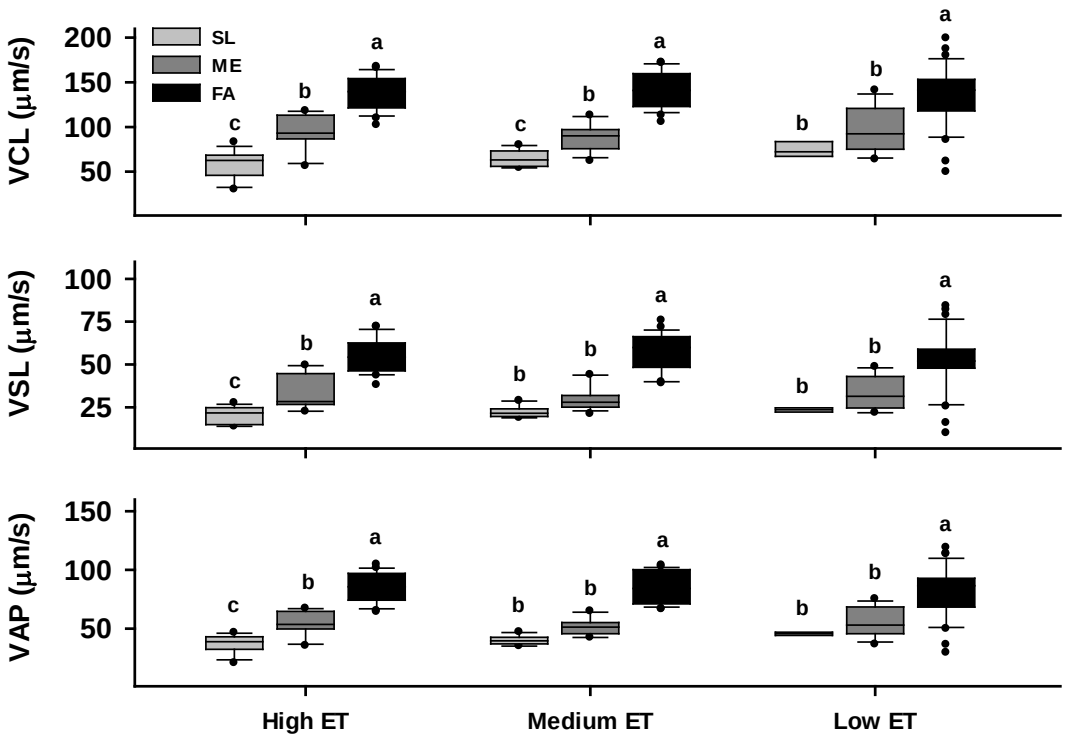

SL
ME
FA
VCL (µm/s)
VSL (µm/s)
VAP (µm/s)
200
150
100
50
100
75
50
25
150
100
50
a
b
c
High ET
Medium ET
Low ET

Figure 7

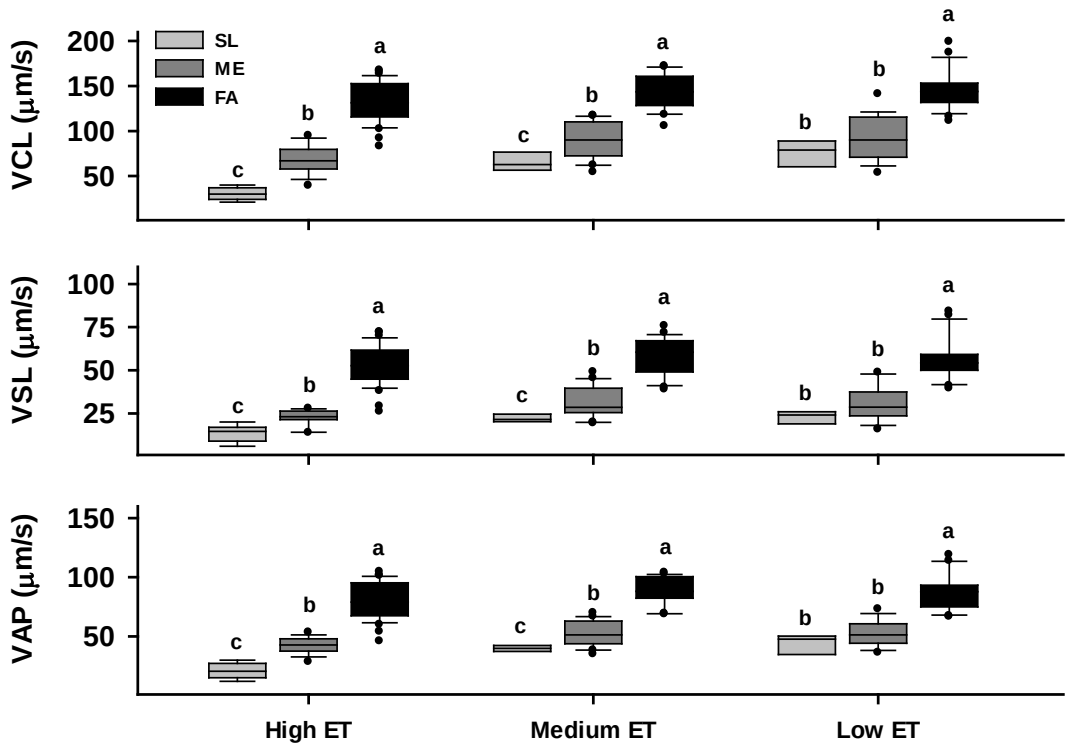


Table 1

			High ET		Medium ET		Low ET	
			MOT Screen	MOT CASA-Mot	MOT Screen	MOT CASA-Mot	MOT Screen	MOT CASA-Mot
C-I	MOT	Micro	0.88	0.92	0.87	0.78	0.88	0.88
	MOT	Screen		0.94		0.93		0.94
C-II	MOT	Micro	0.68	0.65	0.39	0.42	0.49	0.57
	MOT	Screen		0.96		0.87		0.78
C-III	MOT	Micro	0.71	0.86	0.73	0.71	0.79	0.79
	MOT	Screen		0.88		0.66		0.88