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Evaluation of methods to determine sperm density for the European eel, *Anguilla anguilla*

SR Sørensen[1]*, V Gallego[2], L Pérez[2], IAE Butts[1], J Tomkiewicz[1], JF Asturiano[2]

[1]National Institute of Aquatic Resources, Technical University of Denmark, Kavalergården 6, 2920 Charlottenlund, Denmark
[2]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n 46022 Valencia, Spain.

[+] These authors contributed equally to this work

ABSTRACT. European eel, *Anguilla anguilla*, is a target species for future captive breeding, yet best methodology to estimate sperm density for application in *in vitro* fertilization is not established. Thus, our objectives were to evaluate methods to estimate European eel sperm density including spermatocrit, computer assisted sperm analysis (CASA) and flow-cytometry (FCM), using Neubauer Improved hemocytometer as benchmark. Initially, relationships between spermatocrit, hemocytometer counts, and sperm motility were analyzed, as well as the effect of sperm dilution on hemocytometer counts. Furthermore, accuracy and precision of permatocrit, applying a range of G-forces, were tested and the best G-force used in method comparisons. We found no effect of dilution on hemocytometer sperm density estimates, whereas motility associated positively with hemocytometer counts, but not with spermatocrit. Results from all techniques, spermatocrit, CASA and FCM, showed significant positive correlations with hemocytometer counts. The best correlation between spermatocrit and hemocytometer counts was obtained at 6000 × g (r = 0.68). Out of two CASA variants, one or three photographic fields (CASA-1 and CASA-2), CASA-2 showed a very high accuracy to hemocytometer counts (r **=** 0.93), but low precision (CV: CASA-2 =28.4%). FCM was tested with and without microfluorospheres (FCM-1 and FCM-2,) and relationships to hemocytometer counts were highly accurate (FCM-1: r **=** 0.94; 45 FCM-2: r **=** 0.88) and precise (CV: FCM-1 = 2.5; FCM-2 = 2.7%). Overall, CASA-2 46 and FCM-1 feature reliable methods for quantification of European eel sperm, but FCM-1 has a clear advantage featuring highest precision and accuracy. Together, these results provide a useful basis for gamete management in fertilization protocols.

Keywords: sperm density, eel reproduction, European Eel, Anguilla anguilla

* Corresponding author: srs@aqua.dtu.dk

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Abridged title: European eel sperm density

Authors: SR Sørensen[1]*, V Gallego[2], L Pérez[2], IAE Butts[1], J Tomkiewicz[1], JF Asturiano[2]

[1]National Institute of Aquatic Resources, Technical University of Denmark

Kavalergården 6, 2920 Charlottenlund, Denmark.

[2]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal.

Universitat Politècnica de València. Camino de Vera s/n 46022 Valencia, Spain.

* Corresponding author

Phone +45 21314983

Fax: +45 35883434

Email: srs@aqua.dtu.dk

Contents

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We found no effect of dilution on hemocytometer sperm density estimates, whereas motility associated positively with hemocytometer counts, but not with spermatocrit. Results from all techniques, spermatocrit, CASA and FCM, showed significant positive correlations with hemocytometer counts. The best correlation between spermatocrit and hemocytometer counts was obtained at 6000 × g ($r = 0.68$). Out of two CASA variants, one or three photographic fields (CASA-1 and CASA-2), CASA-2 showed a very high accuracy to hemocytometer counts ($r = 0.93$), but low precision (CV: CASA-2 = 28.4%). FCM was tested with and without microfluorospheres (FCM-1 and FCM-2,) and relationships to hemocytometer counts were highly accurate (FCM-1: $r = 0.94$; FCM-2: $r = 0.88$) and precise (CV: FCM-1 = 2.5; FCM-2 = 2.7%). Overall, CASA-2 and FCM-1 feature reliable methods for quantification of European eel sperm, but FCM-1 has a clear advantage featuring highest precision and accuracy. Together, these results provide a useful basis for gamete management in fertilization protocols.

Introduction

European eel, *Anguilla anguilla,* is a well-known species in aquaculture with a commercial value in 2010 of ~8.3 € per kg and production approaching 7000 tons (FIGIS 2012). Still, the eel farming industry relies solely on wild-caught juveniles for production, as protocols for commercial production of glass eels are not available. Since 2006, new integrated methods have expanded this research field for European eel, thus enabling researchers to produce multiple batches of competent gametes, embryos and yolk sac larvae (Tomkiewicz 2012; PRO-EEL 2013).

For several species of marine finfish, it is challenging to produce high-quality gametes for fertilization (Bobe and Labbé 2010). As such, research has focused on how to optimize fertilization strategies for a given species (Butts et al. 2012; 2009). Standardizing the sperm to egg ratio is one such technique that has been used to improve fertilization rates (Bart and Dunham 1996; Christopher et al. 2010; Suquet et al. 1995). Generally lowering the sperm density reduces the fertilization percentage, but any excess sperm sticking to the egg chorion serves as a substrate for microbial activity, which is known to impair embryonic development (Bergh et al. 1992; Oppenheimer 1955). Determining the optimal sperm to egg ratio (among other methods) is therefore important for successful *in-vitro* fertilization, thus implying the need for accurate and precise methods for quantification of sperm concentration and density.

Sperm quality is commonly assessed using density and motility/velocity. In the literature sperm density and motility has been correlated to motility (Rideout et al., 2004). Quantifying spermatozoa density is routinely done by counting the number of spermatozoa in a specific volume of ejaculate (Alavi et al. 2008). The most common counting method is performed using a hemocytometer, which is classified by the World Health Organization as the “gold standard” for sperm quantification in humans (WHO

1999). This method however, is time consuming (Suquet et al. 1992), and precision relies on skilled personnel. As such, studies have been conducted to discover faster and more automated counting methods (reviewed in Fauvel et al. 2010).

Spermatocrit, defined as the ratio of packed sperm to the total volume of milt × 100, is a fast and easy method to estimate spermatozoa concentration. Positive significant correlations between spermatocrit and sperm density estimates, using a hemocytometer, have been reported for several species (Agarwal and Raghuvanshi 2009; Ciereszko and Dabrowski 1993; Hatef et al. 2007; Rideout et al. 2004). However, it is important to note that sperm sedimentation is a reported feature in marine fish species (Fauvel et al. 2010), potentially compromising the accuracy of spermatocrit estimates. In addition, fluctuations in spermatozoa size during the spawning season potentially bias and influence spermatocrit values; for instance, spermatozoa head size changes in marine fish during a spawning season, such as in Atlantic cod (Butts et al. 2011).

Computer assisted sperm analysis (CASA) automates sperm quality assessment, which in turn provides quick, precise, and objective results (Fauvel et al. 2010; López Rodríguez et al. 2011). The strength of CASA lies in quantification of motility, velocity, and behavioral trajectories (i.e. linearity, amplitude of lateral head movement). CASA is furthermore capable of quantifying density of sperm as shown by (Ehlers et al. 2011) together making it a versatile descriptor of sperm quality. Flow-cytometry (FCM) is another automated technique that is able to measure the amount of one or more fluorescent stains in a cell. It features high precision, sensitivity, accuracy, and speed (Cordelli et al. 2005) and due to this deemed a potentially valuable method for assessing male germ cell quality (Cordelli et al. 2005). Within this context, there is a need to assess the applicability of these automated counting methods for the European eel.

100 Spermatogenesis in eels applied in captive reproduction experiments is induced using
101 human chorionic gonadotropin (hCG) (Pérez et al. 2000; Tomkiewicz et al. 2011).
102 Spermiation in European eel starts around week 5 using 1.5 to 2.0 IU hCG g^{-1} fish in
103 weekly treatment (Asturiano et al. 2006; Pérez et al. 2000) with sperm volume
104 increasing until week 8-12 of treatment after which it stabilizes (Asturiano et al. 2006;
105 Tomkiewicz et al. 2011). At this stage, spermatozoa densities are in the range of 5 to 18
106 $\times$ 10^9 cells mL^{-1} (Gallego et al. 2012; Pérez et al. 2000). During spermatozoa
107 maturation, spermatozoa size changes in European eel (Asturiano et al. 2006; Marco-
108 Jiménez et al. 2006). This includes an increase in spermatozoa head length from the 5^{th}
109 to 7^{th} week and head thickening continuing until the 8^{th} week of hormonal treatment
110 (Asturiano et al. 2006; Marco-Jiménez et al. 2006). After the 8^{th} week, only minor
111 changes in spermatozoa/sperm cells head size occur, followed by a decrease in head
112 length from the 12^{th} week and onwards (Marco-Jiménez et al. 2006; Peñaranda et al.
113 2010; Pérez et al. 2009). Within the last decade, European eel sperm have been
114 analyzed using CASA techniques to describe motility parameters (Gallego et al. 2013;
115 Peñaranda et al. 2010; Pérez et al. 2009), ratio of viable spermatozoa (Asturiano et al.
116 2005; 2004) and their morphology (Marco-Jiménez et al. 2006). Furthermore,
117 spermatocrit (12,000 $\times$ g) has been used to standardize sperm:egg ratios in European eel
118 fertilization experiments (Tomkiewicz 2012). However, no studies have been conducted
119 to quantify eel sperm density using CASA or FCM; nor has the accuracy and precision
120 of different methods to quantify sperm density been evaluated.
121 The purpose of this study was to provide fast and reliable tools to measure sperm
122 density for European eel. More specifically, our objectives were to (i) test the
123 relationship between spermatocrit and Neubauer Improved hemocytometer counts, (ii)
124 test whether spermatocrit and hemocytometer counts correlates with sperm motility

class; (iii) assess the effect of sperm dilution on hemocytometer counts; (iii) test the accuracy of spermatocrit for sperm quantification and identify the G-force for best correlation between spermatocrit and hemocytometer counts; (iv) evaluate accuracy and precision of spermatocrit, CASA, FCM using hemocytometer counts as benchmark; and (v) discuss these results in context of applicability for use in hatchery production of the European eel.

Material and methods

Data collection

Fish and hormonal treatment

Male European eels (n = 43; mean standard length and body weight ± SD: 40 ± 2.6 cm and 124 ± 21 g, respectively) were obtained from a commercial eel farm, Stensgård Eel Farm A/S in Jutland, Denmark (55.655461N : 9.20051E). Age of the fish ranged from 2 to 6 years. The fish were transported to a research facility (55.407444N : 9.403414E) of the Technical University of Denmark (DTU) in September 2011, and acclimatized to saltwater over a 10 day period. While at DTU, the eels were kept in 300 L tanks equipped with a closed re-circulation system. The salinity and temperature of the system ranged from 36.7 to 37.3 ppt and 19.5 to 20.5 °C, respectively. Saltwater was made artificially using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany). Fish were maintained under a 12 L light photoperiod at ~20 lux and 12 h dark with a 30 min gradual transition. No feed was provided during the experiment to mimick nature, as eels cease feeding in the silvering stage (Dollerup and Graver 1985).

Hormonal treatment was initiated on 22 September 2011. Prior to onset of hormonal treatment, all males were anesthetized using ethyl p-aminobenzoate at 20 mg L^{-1} (benzocaine; Sigma-Aldrich Chemie, Steinheim, Germany). Each fish was tagged with

150 a passive integrated transponder (PIT tag) in the dorsal muscle tissue. Each week, fish
151 were weighed and received dorsal injections of recombinant human chorionic
152 gonadotropin at 1.5 IU g^{-1} fish (rhCG; Ovitrelle, Madrid, Spain) following Gallego et al.
153 (2012).
154
155 *Sperm sampling*
156 Milt was collected after the 8th (trail 1) and 9th (trials 2+3) hormonal treatment,
157 coinciding with the recommended time to strip sperm for high quality gametes
158 (Asturiano et al. 2006). Sperm samples were obtained 24 h after injection of rhCG to
159 optimize sperm quality (Pérez et al. 2000). Prior to harvest, males were anesthetized
160 using benzocaine, as above. The urogenital pore was thoroughly cleaned using Milli-Q
161 water and dried prior to sperm collection. The first ejaculate of milt was omitted to
162 avoid urine and feces contamination. Ejaculated milt was kept in sterilized 50 mL
163 Falcon tubes, covered using Parafilm®M, and stored at 4 ˚C until motility estimation
164 (max. 30 min). Following motility estimation sperm was refrigerated at 4 ˚C until
165 further assessment (within 5 h).
166
167 *Sperm dilution*
168 Dilutions used for hemocytometer counting, CASA, and FCM were 1:1000 or 1:2000
169 (see below). Hemocytometer counts were performed on fresh sperm, while the other
170 treatments were conducted on preserved sperm samples. Sperm dilutions were done
171 immediately after milt collection in P1 medium (Peñaranda et al. 2010) containing
172 glutaraldehyde 2.5% (v/v) (Sigma-Aldrich Chemie, Steinheim, Germany) to avoid
173 movement of sperm. Dilutions were done using a two-step procedure by first diluting
174 sperm 1:20 and subsequently 1:50 or 1:100 to obtain final dilutions of 1:1000 or 1:2000,

175 respectively.

176

177 *Sperm motility determination*

178 Immediately after milt collection, sperm motility was assessed by mixing 2 µL of milt
179 with 200 µL of 37 ppt artificial seawater (Aqua Medic Sea salt, GmbH, Bissendorf,
180 Germany), with 2% w/v Bovine Serum Albumin (Sigma-Aldrich, Chemie, Steinheim,
181 Germany), adjusted to 8.2 pH (Peñaranda et al. 2010). After activation, 2 µL of sperm
182 were assessed in a SpermTrack-10® chamber (Proiser R+D, S.L.; Paterna, Spain) and
183 observed between 15 and 30 s after activation using a Nikon Eclipse 55i microscope
184 (Nikon Corporation, Tokyo, Japan), fitted with a Nikon DS-Fi1 camera head, and 100×
185 magnification (10× CFI Plan Flour). All the samples were performed in triplicate and
186 analyzed by the same trained observer to avoid subjective differences in motility
187 evaluation. Motility of each replicate was characterized to the nearest 10% increment,
188 averaged, and then categorized into an arbitrary scale where 0: represents no motile
189 sperm; while I: <25%; II: 25-50%; III: 50-75%; IV: 75-90%; and V: 90-100% represent
190 per cent of motile spermatozoa (Pérez et al. 2009).

191

192 *Spermatocrit*

193 Spermatocrit, defined as the ratio of packed sperm to the total volume of milt × 100,
194 was used to estimate sperm concentration. Fresh milt from each male was drawn into
195 three Vitrex™ micro-hematocrit tubes, 75 mm long, with a 1.1 to 1.2 mm opening and
196 sealed using Vitrex™ Sigillum wax. Tubes were centrifuged (Haematokrit 210, Andreas
197 Hettich GmbH & Co.KG, Tuttlingen Germany) for 10 min at specific G-forces ranging
198 from 500 to 14,000 × g (see below for further details). The mean of three measurements
199 per male was used for statistical analyses. Spermatocrit was determined using a digital

caliper (± 0.05 mm).

Hemocytometer counting

A Neubauer Improved hemocytometer was used for counting sperm cell density diluted at 1:1000 or 1:2000 (see section *Sperm dilution*). Sperm counts were done in triplicate and results expressed as spermatozoa $\times 10^9$ mL^{-1}

CASA counting

Milt samples preserved and diluted at 1:2000 in P1 medium (see section *Sperm dilution*) were used for CASA counting. Sperm (2.5 μL) were added to the SpermTrack-10® chamber (Proiser R+D, S.L.; Paterna, Spain) and density was assessed by the concentration module of the Integrated Semen Analysis System (ISAS; Proiser R+D, S.L.; Paterna, Spain). Images for CASA analyses were captured using a Nikon Eclipse E-400 microscope (Nikon Corporation, Tokyo, Japan) equipped with a 10× negative phase objective lens. The image captured represented ~90% of the whole microscope field. The mean number of cells per field varied between 15 and 45 sperm, depending on sperm density. All analyses were performed in triplicate and two different methods were used: CASA-1 = capturing one microscope field per replicate and CASA-2 = capturing three microscope fields per replicate.

Flow cytometer counting

Milt samples used for flow cytometer analyses (Cytomics FC500; Beckman Coulter, USA) were diluted at 1:2000 in P1 medium (see section *Sperm dilution*). Two different methods were applied to calculate sperm density: FCM-1 = at least 5000 events (spermatozoa detected, after discarding debris) were analyzed by a medium flow rate

(30 μL/min) with time as the measured factor in each sample; and FCM-2 = a known concentration of fluorospheres (Flow-Check™ Fluorospheres, Beckman Coulter) were diluted in each sperm sample and at least 5000 events (spermatozoa and fluorospheres detected, after discarding debris) were analyzed by a medium flow rate. Here the ratio of sperm cells/fluorospheres was the registered factor in each sample. In both methods, sperm density was determined by the number of spermatozoa per volume analyzed for each sample. All spermatozoa were stained using 0.1 μM SYBR-14 for 10 min, making sperm distinguishable from the remaining particles. We used a 20-mW air-cooled Argon ion laser with excitation wavelength of 488 nm, and measured emission light using the FL1 photodetector channel to read the green light (525 nm).

Experimental design

Trial 1: Relationships between spermatocrit, sperm density, and motility

Males (n = 43) were stripped and spermatocrit was measured in triplicate for individual males by centrifuging at 12,000 × g for 10 min. Sperm samples were counted using a hemocytometer with a dilution of 1:1000. Sperm motility was assessed for each male.

Trial 2: Effect of sperm dilution

In total, 14 randomly chosen males were stripped and sperm from six of these individuals were selected to have a good dispersion of motility values and avoid bias (10 to 45%). For hemocytometer counts, sperm samples from the same males were diluted at 1:1000 and 1:2000 in P1 medium.

Trial 3: Identification of the optimal G-force

Initially milt from 35 mature males was collected. From these fish, sperm from 10 males were selected covering the range from low to high (27 to 95%) spermatozoa motility. Spermatocrit was measured using 500; 2000; 4000; 6000; 8000; 10,000; 12,000; and 14,000 × g at a centrifugal time of 10 min. For each G-force, new aliquot samples of sperm were used. For each male, hemocytometer counts were obtained using samples diluted at 1:2000 (see section *Hemocytometer counting*).

Trail 4: Test accuracy of automated methods (CASA, FCM) with hemocytometer counts

Data were collected using the same 10 sperm samples as in Trial 3. Automated counting was performed using CASA (CASA-1 and CASA-2) and FCM (FCM-1 and FCM-2). In addition, sperm were counted using a hemocytometer. Measurements were done in triplicate.

Statistical analyses

Data were analyzed using Sigmaplot v. 11 (Systat Software Inc, Hounslow, UK), and R (R Core Team, 2012, Vienna, Austria). Shapiro-Wilk and Levene´s test were used to check for normality and homoscedasticity assumptions, respectively. Data were expressed as mean ± SD. Alpha was set at 0.05 for main effects and interactions.

Trial 1: Relationships between spermatocrit, sperm density, and motility

To compare spermatocrit and hemocytometer counts Model II linear regression was used (ordinary least products regression as described by (Ludbrook 2010)) due to possible variation on both x and y-axes. Model II regression was run for all males and also for a subset of males exhibiting motility values greater than 80%. Furthermore, one-way ANOVAs were run to test whether spermatocrit and hemocytometer counts

were independent of sperm motility class. Hemocytometer data violated ANOVA assumptions. As such, a Kruskal-Wallis test was used for further analyses.

Trial 2: Effect of sperm dilution on sperm density

A student T-test was used to compare sperm density estimates in samples diluted in the ratios 1:1000 and 1:2000, respectively.

Trial 3: Identification of the optimal G-force

Model II linear regression was used to compare hemocytometer counts and spermatocrit for each G-force.

Trial 4: Test accuracy of automated methods (CASA, FCM) with hemocytometer counts

Model II linear regression was used to compare CASA-1, CASA-2, FCM-1, FCM-2, spermatocrit with hemocytometer counts. Next, coefficient of variation (CV) was used for each counting technique to assess between subject variability; spermatocrit values for this analysis were obtained from Trial 3.

Results

Trial 1: Relationships between spermatocrit, sperm density, and motility

Spermatocrit at 12,000 × g ranged from 12.3 to 100% and hemocytometer counts ranged from 1.4 to 21.4 × 10^9 sperm mL^{-1} (Fig. 1). For these 43 males, there was a significant positive relationship between spermatocrit and hemocytometer counts ($r = 0.53$, $F_{1,42} = 15.60$, $P < 0.001$, $y = -1.564 + 4.031x$). However, a high degree of scatter was observed in the spermatocrit values; i.e. spermatocrit values for hemocytometer

counts around 8 x 10^9 mL^{-1} ranged from 15 to 60%. The hemocytometer counts for males showing motility > 80% (n = 10) were generally higher, resulting in a different relationship between spermatocrit and hemocytometer counts ($r = 0.62$, $F_{1,9} = 5.02$, $P = 0.030$, $y = -24.434 + 4.661x$).

[Insert Figure 1]

Hemocytometer counts were associated with motility class, such that sperm counts were significantly higher in motility class V (approaching 100 %) than in class 0 with lowest motility ($F_{4,37} = 2.73$, $P = 0.034$; Fig. 2). On the contrary, spermatocrit values did not vary among sperm motility classes ($H = 4.789$, $P = 0.442$; Fig. 2); class 0 showed high variability as it was composed of two individuals.

[Insert Figure 2]

Trial 2: Effect of sperm dilution on sperm density

The effect of dilution (1:1000 vs. 1:2000) on hemocytometer estimates of sperm density was non-significant ($t_{10} = 0.048$, $P = 0.963$; Fig. 3); only the variation among replicates tended to be higher at lower dilution.

[Insert Figure 3]

Trial 3: Identification of optimal G-force

Sperm from Male 3 and Male 8 showed a rapid decrease in spermatocrit over the G-force gradient (Fig. 4). There were significant positive relationships between

spermatocrit and hemocytometer counts at 500; 4000; 6000; 12,000; and 14,000 × g (r values ranged from 0.33 to 0.68, $P \leq 0.049$; Fig. 5.). The best relationship was found between spermatocrit and hemocytometer counts at 6000 × g ($r = 0.68$, $P = 0.016$; Fig. 5), as such these G-force data were used for further comparisons.

[Insert Figure 4]

[Insert Figure 5]

Trail 4: Test accuracy of automated methods (CASA, FCM) with hemocytometer counts

CASA-1 ($r = 0.70$, $F_{1,9} = 7.61$, $P = 0.012$) and CASA-2 ($r = 0.93$, $F_{1,9} = 51.16$, $P < 0.001$; Fig. 6) density estimates were positively related to hemocytometer counts. Furthermore, there were significant positive relationships between FCM-1 ($r = 0.94$, $F_{1,9} = 62.921$, $P < 0.001$) and FCM-2 ($r = 0.88$, $F_{1,9} = 26.84$, $P < 0.001$) and hemocytometer counts.

[Insert Figure 6]

The CVs for CASA-1 (17.9%) and CASA-2 (28.4%) were in the order of 7.5 times greater compared to the other counting techniques (CV ranges from 2.5 to 5.9%; Table 1).

[Insert Table 1]

Discussion

In this study, we report several key findings: (i) hemocytometer counts were positively associated sperm motility; (ii) hemocytometer counts were not affected by milt dilution

ratio; (iii) optimizing G-force for centrifuging milt improved the relationship between spermatocrit and hemocytometer counts; (iv) spermatocrit, CASA and FCM, were all positively related to hemocytometer counts with CASA-2 and FCM-1 having the strongest relationship to hemocytometer counts.

Spermatocrit has been used to estimate sperm concentration for several species of fish (Rakitin et al. 1999; Rideout et al. 2004), such as yellow perch, *Perca flavescens* (Ciereszko and Dabrowski 1993), haddock, *Melanogrammus aeglefinus* (Rideout et al. 2004), Atlantic halibut, *Hippoglossus hippoglossus* (Tvedt et al. 2001), snow trout, *Schizothorax richardsonii* (Agarwal and Raghuvanshi 2009), brown trout, *Salmo trutta* (Poole and Dillane 1998), Atlantic salmon, *Salmo salar* (Aas et al. 1991), rainbow trout, *Oncorhynchus mykiss* (Ciereszko and Dabrowski 1993) and lake whitefish, *Coregonus clupeaformis* (Ciereszko and Dabrowski 1993). Together these studies found spermatocrit as a quick and easy technique for estimating sperm concentration (Alavi et al. 2008). In the present study, we evaluated the relationship between spermatocrit and hemocytometer counts for the European eel and showed a significant positive relationship between these two quantitative sperm metrics. However, its relationship with hemocytometer counts showed considerable scatter and appeared inferior to the automated counting methods. Furthermore, the tests of different centrifugal G-forces revealed that r-values varied between 0.33 and 0.68 and the best relationship between spermatocrit and hemocytometer counts was obtained at 6000 × g. Higher centrifugal forces tended to result in low correlation coefficients, as a result of changes in cell packing within the microhematocrit tube.

A non-significant relationship between spermatocrit and hemocytometer counts was found in Atlantic cod, *Gadus morhua* (Rakitin et al. 1999). The authors suggested this might be an artifact of small volumes of milt being diluted in immobilizing media

374 before sperm density was quantified using a hemocytometer. This study by Rakitin et al.
375 (1999) used a one-step 500-fold dilution and their reported variability was high (CV =
376 27.7%). We found negligible effect of milt dilution ratio on hemocytometer counts as
377 well as a low coefficient of variation (CV = 5.9%). The precision and accuracy of
378 hemocytometer counts has been addressed in the literature (see Alavi et al. 2008 and
379 Fauvel et al. 2010 for review) and errors due to pipetting, dilution ratio, sperm settling
380 times, and operator biases are emphasized (Rakitin et al. 1999). Therefore, there is a
381 need for species-specific guidelines for fishes as set by the WHO for humans (WHO
382 1999).

383 Sperm motility and spermatocrit values were independent, while hemocytometer
384 density estimates increased with motility class, such that the low motility class 0 (no
385 motility) had significantly lower sperm density than the high motility class V (90-100%
386 motility). The latter concurs with final hydration of spermatozoa coinciding with final
387 maturation and increase of motility (Gallego et al. 2012). Useful future research should
388 relate these quantitative sperm metrics to other estimates of quality, such as sperm
389 velocity and fertilization success.

390 In our study, CASA-2 and FCM-1 show strong predictive relationships with
391 hemocytometer counts ($r = 0.93$ and 0.94, respectively). FCM-1 gave the strongest
392 relationship. FCM has an advantage over CASA in that it has a 10-fold lower
393 coefficient of variation. Similarly, sperm counts measured by hemocytometer and flow
394 cytometer were also highly correlated ($r^2 = 0.85$) in the razorback sucker, *Xyrauchen*
395 *texanus* (Jenkins et al. 2011). CASA, although not commonly used for quantification of
396 fish sperm density, gave us promising result. This indicates that CASA is a universal
397 tool for sperm quality/quantity assessment and further complements flow cytometry
398 which -besides quantification, can describes the physiology of milt parameters (i.e.

399 membrane potential, cell integrity; Cordelli et al. 2005; Fauvel et al. 2010). CASA
400 software is commonly used throughout the field of sperm biology (Marco-Jiménez et al.
401 2006; Peñaranda et al. 2010; 2008; Pérez et al. 2009), as open-source systems have
402 immerged, resulting in inexpensive alternatives for sperm quality assessment (Komori
403 et al. 2006; Wilson-Leedy and Ingermann 2007). We recommend these automated
404 systems for studying reproductive physiology and for routine assessment of sperm
405 density for the European eel. Additionally, spectrophotometry methods should be
406 examined (Fauvel et al. 1999).

407 When deciding which method to use for quantification of sperm, both economic
408 feasibility and accuracy/precision of specific device(s) need to be considered. In Table
409 2, we provide an overview of resource requirements, advantages, and disadvantages for
410 the different quantitative methods investigated. In summary, the hemocytometer
411 features low operational costs, precise measurements, but is time consuming and
412 precision relies on skilled personnel. Spermatocrit measurements require a centrifuge,
413 low level of operator training, are fast, but are not as accurate as other methods. CASA-
414 1 requires special software and a microscope with video frame grabber. Additionally,
415 CASA-1 gives fast results, but has relatively low accuracy and precision. CASA-2, like
416 the aforementioned, needs software, requires a microscope, and video frame grabber.
417 Furthermore, CASA-2 gives an accurate result, but at low precision. FCM-1 requires
418 expensive equipment, gives both accurate and precise results, while FCM-2 features the
419 same characteristics, although slightly more expensive and less accurate. Both the
420 hemotocymeter and automated counting techniques differ from spermatocrit by giving
421 counts rather than concentration, and therefore are likely less subjective to bias from
422 changes in spermatozoa head morphology (Marco-Jiménez et al. 2006).
423

[Insert Table 2]

In conclusion, we found highly predictive relationships between CASA-2 and FCM-1 and hemocytometer counts, which can be considered as accurate methods for quantification of European eel sperm. These methods appear the most efficient for developing standardized fertilization protocols, enabling optimized sperm to egg ratios. We also found a lower, but significant correlation between spermatocrit and hemocytometer counts, although not as clear as reported in some other fish species.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

SRS, JT and JFA conceived the experiment. SRS, VG, LP and JFA performed the experimental design and experiment execution. SRS, VG, IEAB, JT and JFA performed data analyses and interpretation. JT and JFA supervised the study design, execution, analysis and approved the final version. All authors read and approved the manuscript.

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589

590 **Author's address (for correspondence)**:

591 SR Sørensen,

592 Technical University of Denmark, National Institute of Aquatic Resources.

593 Kavalergården 6, 2920 Charlottenlund, Denmark

594 Email: srs@aqua.dtu.dk

595 Phone: +4521314983

596 Fax: +4535883434

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600 Figure 1

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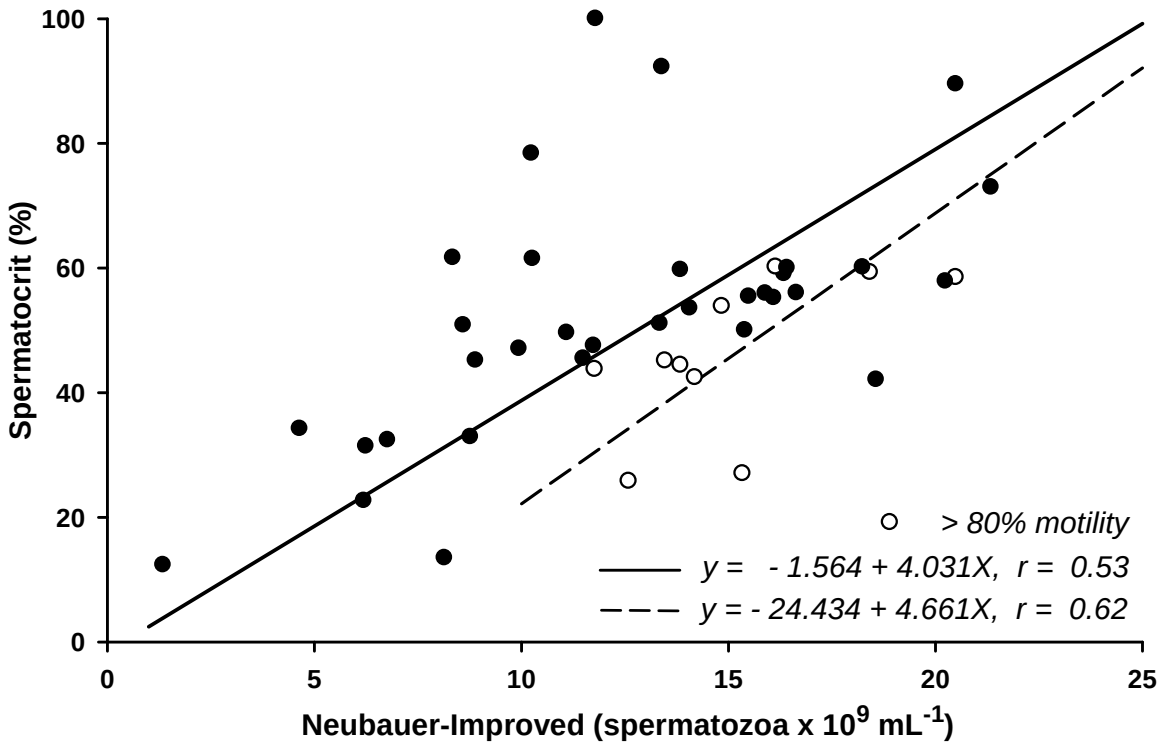

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604 Fig. 1. Relationships between spermatocrit and hemocytometer counts in the European
605 eel, *Anguilla anguilla*. Model II linear regression was used (ordinary least products
606 regression as described by (Ludbrook 2010)) due to possible error in both x and y-axes.
607 Regression analyses were run for all males (n = 43) and this is represented by a solid
608 line; those males with motility >80% (n = 10) are represented by open circles and a
609 dashed line.

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Figure 2

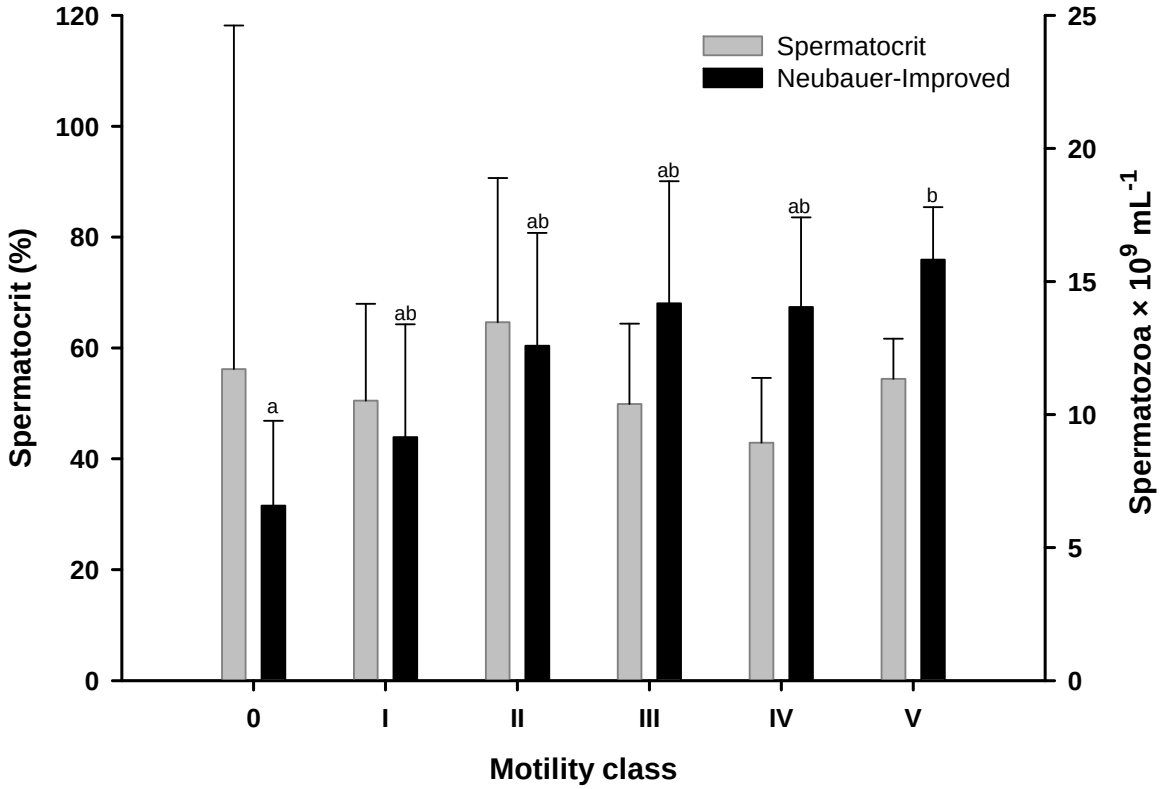

Fig. 2. Spermatocrit (gray bars on primary y-axis) and hemocytometer counts (black bars on secondary y-axis) for five sperm motility classes in the European eel, *Anguilla anguilla*. Data are expressed as mean ± SD. Values with common letters were not significantly different via one-way ANOVA. 0 = 0% motility; I: 1 to 25% motility; II: 25 to 50% motility; III: 50 to 75% motility; IV: 75 to 90% motility; V: 90 to 100% motility.

Figure 3

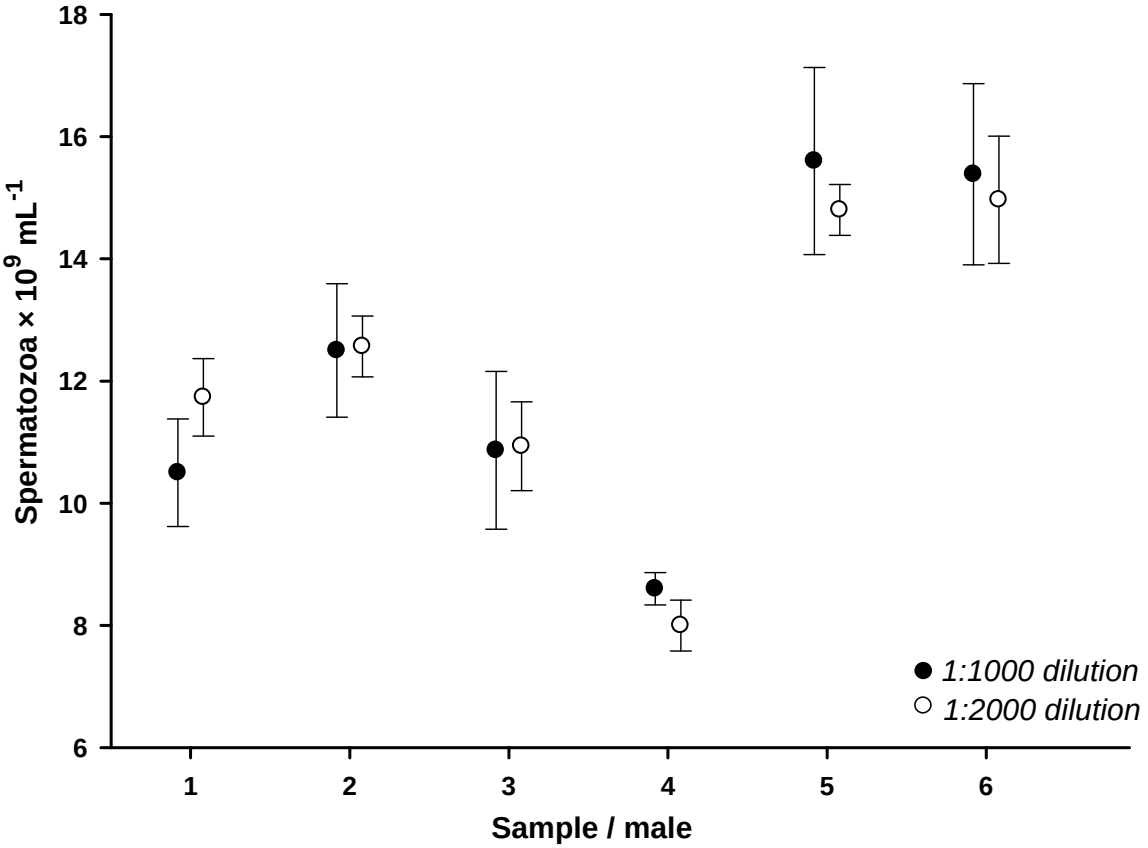

Fig. 3. Hemocytometer counts for six males using two different milt dilutions in the European eel, *Anguilla anguilla*. Solid symbols = 1:1000; open symbols = 1:2000 dilution.

Figure 4

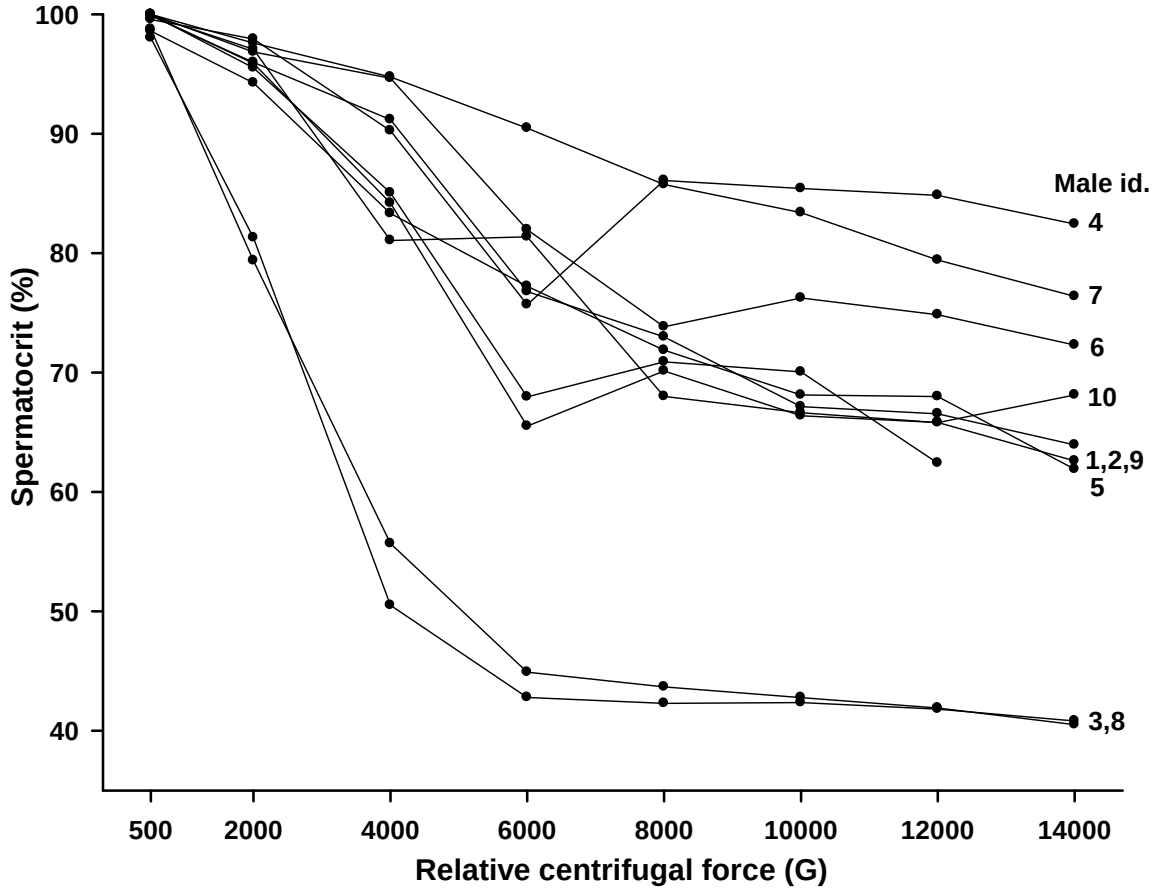

Fig. 4. Values of spermatocrit for 10 males over a G-force gradient (500 to 14,000 × g) in the European eel, *Anguilla anguilla*. Male Id is shown on the right (1 to 10).

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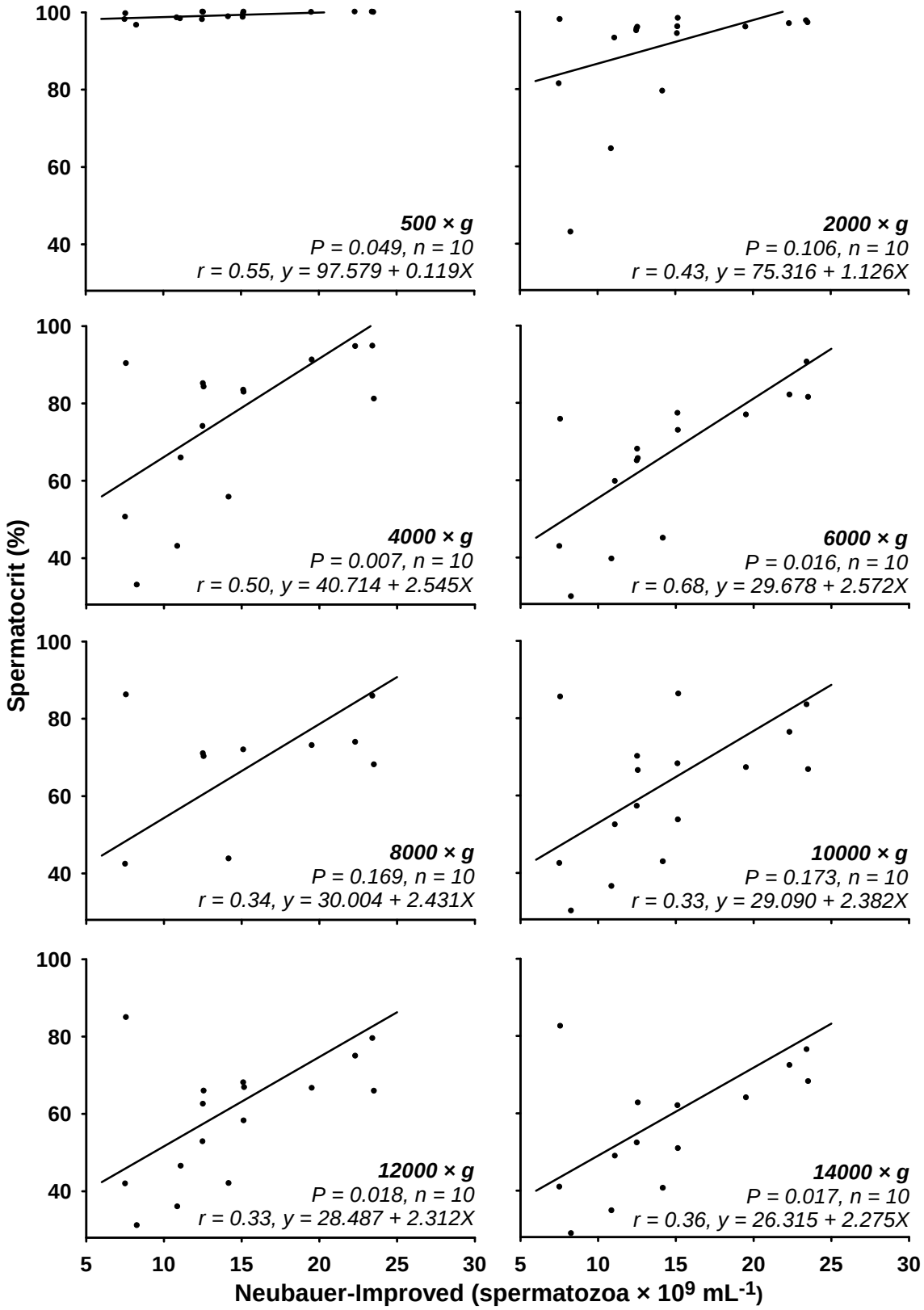
650 Figure 5


Spermatocrit (%)
100
80
60
40
500 × g
P = 0.049, n = 10
r = 0.55, y = 97.579 + 0.119X
2000 × g
P = 0.106, n = 10
r = 0.43, y = 75.316 + 1.126X
4000 × g
P = 0.007, n = 10
r = 0.50, y = 40.714 + 2.545X
6000 × g
P = 0.016, n = 10
r = 0.68, y = 29.678 + 2.572X
8000 × g
P = 0.169, n = 10
r = 0.34, y = 30.004 + 2.431X
10000 × g
P = 0.173, n = 10
r = 0.33, y = 29.090 + 2.382X
12000 × g
P = 0.018, n = 10
r = 0.33, y = 28.487 + 2.312X
14000 × g
P = 0.017, n = 10
r = 0.36, y = 26.315 + 2.275X
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Neubauer-Improved (spermatozoa × 10^9 mL^-1)

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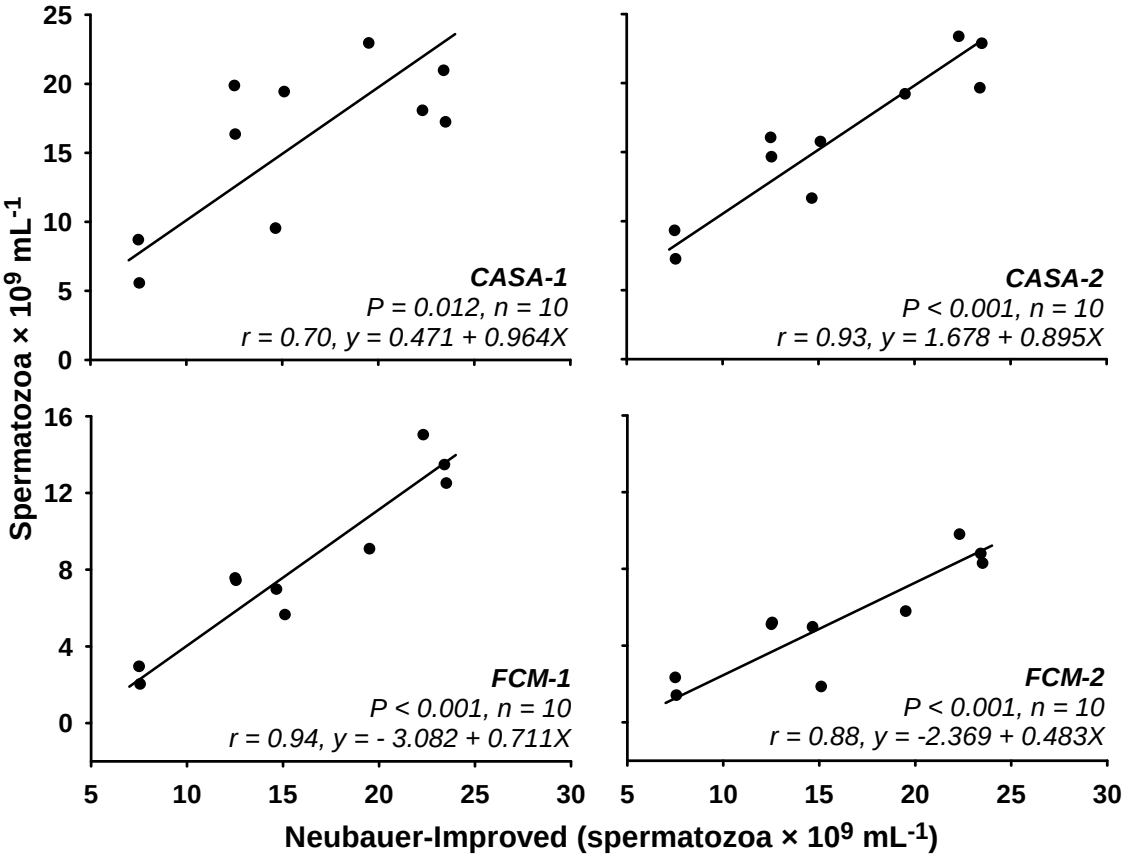
Fig. 5. Relationships between spermatocrit and hemocytometer counts over a G-force gradient (500 to 14000 × g) in the European eel, *Anguilla anguilla*. Model II linear regression was used (ordinary least products regression as described by (Ludbrook 2010)) due to possible error in both x and y-axes. For each plot the P-value, sample size, correlation coefficient, and equation of line are shown.

674 Figure 6



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677 Fig. 6. Relationships between CASA-1, CASA-2, FCM-1, FCM-2 and hemocytometer
678 for the European eel, *Anguilla anguilla*. Model II linear regression was used (ordinary
679 least products regression as described by (Ludbrook 2010)) due to possible error in both
680 x and y-axes. For each plot the P-value, sample size, correlation coefficient, and
681 equation of line are shown.

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685 Table 1. Coefficients of variation for hemocytometer, spermatocrit at 6000 × g,
686 computer assisted sperm analysis (CASA-1 and CASA-2) and flow cytometry (FCM-1
687 and FCM-2) for the European eel, *Anguilla anguilla*. Mean values are shown for each
688 counting method. Measurements were performed in triplicate for 10 males.
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Male number	Neubauer-Improved	Spermatocrit	CASA-1	CASA-2	FCM-1	FCM-2
1	6.1	6.5	36.5	34.7	5.5	3.6
2	11.2	3.0	11.7	31.5	1.7	2.5
3	8.4	2.1	13.8	27.4	1.8	3.5
4	0.0	10.1	6.9	36.0	2.1	1.5
5	5.1	9.6	30.6	27.3	1.7	1.4
6	4.7	6.8	12.0	21.0	2.5	3.9
7	8.7	6.8	1.8	16.0	3.8	4.2
8	6.0	4.0	29.0	21.5	2.0	3.2
9	7.6	4.2	32.4	26.4	0.8	3.0
10	0.9	3.1	3.8	42.2	3.6	0.5
Mean	5.9	5.6	17.9	28.4	2.5	2.7

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703 Table 2. Resource requirements, advantages, and disadvantages for the different
704 quantitative methods used to determine sperm density for the European eel, *Anguilla*
705 *anguilla*.

Quantification method	Requirements	Advantages	Disadvantages
Neubauer-Improved hemocytometer	• microscope required • Neubauer Improved hemocytometer • trained personnel	• cheap • precise - low CV • described in literature	• time consuming
Spermatocrit	• centrifuge required • microhematocrit tubes • tube sealant • haematocrit tube reader	• fast • precise - low CV • low level of training	• inaccurate - low r • sperm sedimentation
CASA-1	• CASA software • software calibration • computer and microscope with frame grabber • training	• fast • additional measures of sperm quality obtained	• low precision - high CV • inaccurate – low r • trained personnel
CASA-2	• CASA software • software calibration needed • computer and microscope with frame grabber • training	• fast • accurate - high r • additional measures of sperm quality easy obtainable	• low precision - high CV trained personnel
FCM-1	• flow cytometer required • training	• precise - low CV • accurate – high r	• trained personnel • need to extrapolate by equation
FCM-2	• flow cytometer and fluorospheres required • training	• precise - low CV • accurate – high r	• fluorospheres making it more expensive than FCM-1 • lower accuracy than FCM-1 • need to extrapolate by equation

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