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Standardization of fertilization protocols for the European eel, *Anguilla anguilla*

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ABSTRACT

Standardization of artificial fertilization protocols for the European eel, *Anguilla anguilla*, is a prerequisite for optimizing the use of available gametes in hatchery facilities and for conserving sperm from high quality males, which is either cryopreserved or in living gene banks. The objectives of this research were to provide a rapid, accurate and precise method to quantify sperm density by examining the relationship between sperm density and absorbance by use of a spectrophotometer, determine the optimal number of sperm required to fertilize eggs in a controlled setting, and explore how long eggs are receptive to fertilization post-stripping. Mean sperm density and absorbance at 350 nm were 1.54e + 10 ± 4.95e + 9 sperm/mL and 1.91 ± 0.22 nm, respectively. Regression analysis demonstrated a highly significant positive relationship between sperm density and absorbance using a spectrophotometer at 350 nm ($R^2 = 0.94$, $p < 0.001$, $y = 2.273e + 10x - 2.805e + 10$); significant but slightly weaker relationships were also detected at 400, 500, and 600 nm ($R^2 \geq 0.93$, $p < 0.001$). Fertilization success using sperm to egg ratios ranging from 1.3e + 3 to 1.0e + 6 sperm per egg increased from 37.5 to 68.1%, respectively. Sperm to egg ratio had a significant effect on fertilization success ($p < 0.0001$), where fertilization success increased from 1.3e + 3 to 2.5e + 4 sperm per egg; adding greater than 2.5e + 4 sperm per egg had no significant effect. Furthermore, the duration of time post-stripping had a significant effect on egg fertilization success ($p < 0.0001$), such that between 0 and 10 min post-stripping 57.4 to 78.2% of the eggs were fertilized while at 15 min post-stripping a significant decrease in fertilization success was detected (47.5%). For all statistical models, the female variance component was significant for fertilization success ($p < 0.0001$) and explained ≥84% of the models variance. In conclusion, European eel eggs should be fertilized within 10 min post-stripping using 2.5e + 4 sperm per egg. Together, these findings will contribute to the development of European eel breeding technology and further our understanding on sperm biology and reproductive biology in fishes.

1. Introduction

European eel, *Anguilla anguilla*, has long been a highly valued species targeted for aquaculture production (Gousset, 1990; Ottolenghi et al., 2004). Nevertheless, the eel farming industry relies exclusively on wild-caught juveniles, as rearing protocols for commercial production of glass eels (an intermediary stage in the eel's life history) are not yet available. Recently, pioneering research has raised eel breeding from a state of reproductive failure to a stable production of yolk-sac larvae (reviewed in Tomkiewicz et al., 2012). Although basic procedures for artificial fertilization have been described (Mordenti et al., 2013; Tomkiewicz et al., 2012), as of yet no empirical research has been directed towards standardizing fertilization protocols. This is especially critical for species whose gametes are hand-stripped, such as the European eel, as it may increase fertilization success and embryonic survival.

Before standardizing fertilization protocols, for any given species, there is a need for accurate and precise methods to quantify sperm density. Currently, several methods are available to quantify sperm density for the European eel (Sørensen et al., 2013). In brief, haemocytometer counting featured low operational costs, high precision and accuracy, but was a tedious and time-consuming technique. Spermatocrit measurements were cost-effective and rapid, but not as accurate as the other methods, while computer-assisted sperm analysis and flow cytometry offered fast and unbiased estimation, but required expensive software/equipment and skilled personnel (Sørensen et al., 2013). Determination of sperm density by use of a spectrophotometer however, has yet to be explored for this species. This method is known to be reliable, rapid, simple, and inexpensive, thus would allow for more efficient use of time and prevent stripped gametes from standing too long before fertilization (Alavi et al., 2008; Dong et al., 2005).

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Fertilization success is predicted to increase when more sperm are incorporated into an artificial and/or natural spawning event (Butts et al., 2009; Hatef et al., 2009). However, the benefit of sperm number should decrease as the total number of cells approaches the number required to fertilize an entire egg batch (Ball and Parker, 1996; Casselman et al., 2006). Excess sperm sticking to the chorion of adhesive eggs can also serve as a substrate for microbial activity, which is known to impair embryonic development until hatching (Bergh et al., 1992). Additionally, two or more sperm entering the egg (termed polyspermy) may lead to abnormal embryonic development and ultimately to death of an embryo, even though mechanisms exist to minimize and/or block polyspermy in vertebrates and invertebrates (Psenicka et al., 2010; Snook et al., 2011). Therefore, determining the "optimal" sperm to egg ratio is a critical step towards establishing successful in vitro fertilization protocols.

Gamete receptivity, defined as the length of time gametes are receptive to be fertilized, also impacts reproductive yields and is another factor which should be considered when spawning fishes in captivity (Butts et al., 2012a). For instance, when European eel sperm are activated they lose their fertilization capability within 1–2 min due to limited amounts of energy reserves available for motility, whereas non-activated cells can be stored in a refrigerator for extended periods and still maintain a high capacity to fertilize eggs upon activation (~80% motility at 7 days of storage; Peñaranda et al., 2010). To the best of our knowledge, it is unclear how long European eel eggs are receptive to fertilization post-stripping. In fishes, eggs of high quality, are usually viable for only a restricted time post-stripping, but may be prolonged at appropriate temperatures (Ciereszko et al., 2000). Therefore, defining the trajectory of this decline is important to optimize spawning collection procedures for aquaculture and/or research activities (Johnston et al., 2008).

Therefore, the main goal of this study was to standardize artificial fertilization protocols for the European eel. More specifically, the objectives were to (i) develop a relationship between sperm density and absorbance by use of a spectrophotometer, (ii) use this newly developed relationship to determine the optimal number of sperm required to fertilize eggs in a controlled setting, and (iii) explore how long eggs are receptive to fertilization post-stripping. Overall, these findings will contribute to the development of European eel aquaculture and further our understanding on sperm biology and reproductive biology in fishes.

2. Materials and methods

2.1. Broodstock collection and management

Female broodstock (n = 42; mean standard length and body weight ± SEM were 68.8 ± 1.4 cm and 669.0 ± 47.7 g, respectively) were caught in the silvering stage from a freshwater lake (Vandet Sø) in northern Jutland, Denmark. Eels were transported, using an aerated transportation tank, to a research facility (55.407444 N: 9.403414E) of the Technical University of Denmark (DTU). While at DTU, eels were injected with a passive integrated transponder (PIT tag) in the dorsal muscle and housed in 300 L tanks equipped with a closed re-circulation system. Acclimatization to saltwater took place over a 14-day period. Salinity was adjusted artificially using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany). During maturation, eels were kept at a density of ≤30 kg per m^3, salinity of ~36‰, and temperature of ~19 to 21 °C. Eels were maintained under dimmed light conditions at ~20 lx and a natural daily photoperiod with gradual transition taking 30 min. No feed was provided during experimentation as eels in silvering stage cease feeding (Dollerup and Graver, 1985).

To induce vitellogenesis females received weekly (10 to 20 weeks) injections of salmon pituitary extract (SPE; 18.75 mg/kg body weight, Argent Chemical Laboratories, Washington, USA) (Kagawa et al., 2005). Biopsies were routinely taken for evaluation of oocyte development (Palstra et al., 2005). Based on body weight increase and oocyte developmental stage indices, females received another injection of SPE as a priming dose. To stimulate final maturation and induce ovulation females were later injected, ~24 h after receiving the priming dose, with the maturation-inducing steroid (MIS; 17α,20ß-dihydroxy-4-pregnen-3-one; 2 mg per/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) (Ohta et al., 1996). Within 12 to 14 h of receiving MIS, eggs were stripped by applying pressure to the abdomen of the fish. Expelled eggs were collected into dry weight boats (7 cm × 7 cm with volume of 80 mL).

Male broodstock were reared in a commercial eel farm (Stensgård Eel Farm A/S) in Jutland, Denmark (55.655461 N: 9.20051E) on a standard diet (DAN-EX 2848, BioMar A/S, Brande, Denmark) (for composition see Støttrup et al., 2013). Males (n = 60; mean standard length and body weight were ± SEM 38.1 ± 0.3 cm and 110.0 ± 2.0 g, respectively) were transported to DTU's research facility (husbandry conditions as above). Male eels received weekly injections of recombinant human chorionic gonadotropin at 1.5 IU g^{-1} fish (Ovitrelle, Madrid, Spain) (Gallego et al., 2012). Milt was collected after 7 weeks of hormonal treatment (~12 h after administration of hormone). For milt collection, the genital pore was wiped dry using deionized water. The first ejaculate of milt was omitted to avoid urine and faeces contamination. Samples were then collected into dry weight boats (3.6 cm × 3.6 cm with volume of 7.5 mL), by applying slight pressure along the abdominal region. Within 10 s, 500 μL of milt from each male was pipetted into 20 mL of immobilizing medium (Peñaranda et al., 2010).

2.2. Sperm motility assessment

Sperm motility was assessed according to Sørensen et al. (2013). In brief, within 30 to 40 min post-stripping, the percentage of motile sperm per male was estimated by adding 0.2 μL of milt onto the centre of a microscope slide, situated on the stage of a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan) maintained at room temperature. Sperm were activated by adding 200 μL of ~20 °C seawater obtained from the North Sea and adjusted to 36 ppt with artificial Red Sea salt (Red Sea Europe, Verneuil sur Avre, France). No coverslip was added during sperm activation. Sperm motility was assessed at 400× magnification within 10 to 15 s after the addition of seawater. Motility was characterized using an arbitrary scale where 0: represents no motile sperm; while I: <25%; II: 25–50%; III: 50–75%; IV: 75–90%; and V: 90–100% represent percentage of motile spermatozoa (Pérez et al., 2009; Sørensen et al., 2013). All samples were performed in triplicate and analyzed by the same trained observer to avoid subjective differences in motility assessment. Activation motility scores were all ≥75% (characterized as stage IV or V); no significant difference in sperm motility was detected between the sperm pools ($p < 0.05$).

2.3. Experiment 1: Determination of sperm density by use of a spectrophotometer

Milt (50 μL) was collected from 18 males and each sample was diluted into disposable glass test tubes (16 mm × 100 mm, Fisher Scientific, Loughborough, UK) containing 3000 μL of immobilizing media (P1 media as described in Peñaranda et al., 2010). The resulting sperm suspensions were homogenized using a vortex mixer for 10 s (Minishaker MS2; IKA, Staufen, Germany). Homogenized samples were immediately transferred to plastic cuvettes, the outside of each cuvette was wiped with Kimwipes®, and then placed into a spectrophotometer (DR 2800; Hach-Lange Aps, Brønshøj, Denmark). Absorbance values were randomly generated across four different wavelengths spanning the visible spectrum: 350, 400, 500 and 600 nm. The mean of three replicates per male was used for statistical analyses.

Sperm density was counted under a Zeiss Axiostar compound microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) at 400× magnification using an improved Neubauer haemocytometer (see

Butts et al., 2012b for details). Homogenized milt (200 μL) from the plastic cuvettes was further diluted in 3200 μL of immobilizing media and mixed using a vortex mixer for 10 s. A sample of this sperm suspension (10 μL) was then micropipetted onto a haemocytometer that had been pre-covered with a coverslip. The number of sperm in each of the five larger squares on the haemocytometer was counted. There are 25 of these large squares on the haemocytometer and each of these large squares has 16 smaller squares within it. Sperm were counted in the four large corner squares and the large centre square. The mean number of sperm per large square count (i.e. mean of the five counts) was multiplied by 25 (to obtain the mean per 5 × 5 large-square grid), by 10 (the depth of the chamber in mm), and then by the initial volume of the sample to estimate the sperm density. Sperm densities are expressed as the total number of sperm per mL of a male's ejaculate. The mean of three replicates per male was used for statistical analyses.

2.4. Experiment 2: Determination of the optimal sperm to egg ratio

Eggs were collected from six females and milt was stripped from 24 haphazardly selected males. Milt (500 μL) from four males was diluted into 20 mL of immobilizing media to create six unique sperm pools (500 μL of milt × 4 new males = 2000 μL of diluted milt into each sperm pool). Eggs from each female were then "crossed" (see below) with a new sperm pool from the four males.

Using a micropipette, a known volume of sperm from each pool (adjusted according to the calculated sperm density using results outlined for Experiment 1 in Section 3.1) was added to 2 mL microcentrifuge tubes (Sarstedt AG & Co., Nümbrecht, Germany) creating 12 experimental sperm to egg ratios for each sperm pool (1.3e + 3:1, 2.5e + 3:1, 5.0e + 3:1, 1.0e + 4:1, 2.5e + 4:1, 5.0e + 4:1, 7.5e + 4:1, 1.0e + 5:1, 2.5e + 5:1, 5.0e + 5:1, 7.5e + 5:1, 1.0e + 6:1 sperm to egg). Three replicate microcentrifuge tubes were used for each sperm to egg ratio. Each microcentrifuge tube was filled to 1000 μL with immobilizing media. Approximately 500 eggs (mean ± SEM = 506 ± 10 eggs) were placed into dry weight boats (7 cm × 7 cm) using a 1.0 mL syringe. The tip of each syringe was cut off to prevent the eggs from being compressed or damaged. Sperm in each microcentrifuge tube was then added to the eggs in the weight boats. Activation media (20 mL of 37 ppt seawater at ~20 °C) was immediately added to the eggs. After 5 min gamete contact time, eggs were transferred into 250 mL plastic tri-corner beakers containing 200 mL of 36 ppt seawater for incubation. The embryos were incubated at 20 °C until being examined for fertilization success. Fertilization success was determined 3 to 5 h post-fertilization, by examining a mean (±SEM) of 70 ± 2 eggs per replicate. Embryos were observed and images captured using a compound microscope equipped with a digital camera (as above). Fertilization success was calculated as the percent fertilized eggs. Fertilized eggs were identified by the presence of blastomere cleavage (4 to 64 stages), and those not showing >4-cell cleavages were considered unfertilized.

2.5. Experiment 3: Effect of time post-stripping

Eggs from three females were "crossed" with a sperm pool from four males (in total 12 males were used) at 0, 5, 10, 15, 20, 30, 40, 60 min (±3 min) post-egg stripping (see Section 2.4 for further details on fertilization procedures). For each female, three replicate crosses were performed at each time post-egg stripping. Throughout storage, the eggs were held in dry weight boats (without extender media), sealed in plastic bags, and stored in a cooler at 20 °C. Based on results outlined in Experiment 2 (see Section 3.2), crosses were conducted using a 2.5e + 4:1 sperm to egg ratio.

2.6. Statistical analyses

Data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) and RMA software for reduced major axis regression (v. 1.17; http://www.bio.sdsu.edu/pub/andy/rma.html) (Bohonak, 2004). Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Fertilization success was arcsin square-root transformed (Zar, 1996). Alpha was set at 0.05.

2.6.1. Experiment 1: Determination of sperm density by use of a spectrophotometer

Linear regression analysis was used to examine the relationship between sperm density and absorbance by use of a spectrophotometer. Separate regressions were run at each absorbance. One of the underlying assumptions of standard ordinary least squares regression is that the independent variable, or X-axis, is measured with no error (Zar, 1996). Because absorbance was measured with "possible error", data were analyzed using RMA software (Model II regression, Ludbrook, 2010).

2.6.2. Experiments 2 and 3: Determination of the optimal sperm to egg ratio, and effects of time post-stripping

Fertilization success was analyzed using a mixed-model ANOVA (PROC MIXED; SAS Institute, 2003) where sperm to egg ratio was considered a fixed factor and female was considered a random blocking factor. A-posteriori analyses were not performed on random female effects. Instead, variance components were constructed using the restricted maximum likelihood (REML) estimation method in SAS PROC Mixed, and expressed as a percentage. To test for significance among variance components (VC) greater than zero, likelihood ratio statistics were generated (SAS Institute, 2003). Denominator degrees of freedom for all F-tests were approximated using the Kenward-Roger method (Spilke et al., 2005). A-posteriori analyses performed on fixed effects were constructed using Tukey's multiple comparisons procedure.

3. Results

3.1. Experiment 1: Determination of sperm density by use of a spectrophotometer

The overall mean (±SD) sperm density for the 18 males was 1.54e + 10 ± 4.95e + 9 sperm/mL with male mean values ranging from 7.13e + 9 to 2.32e + 10 sperm/mL. Regression analysis demonstrated that the most variance was explained between sperm density and absorbance using a spectrophotometer at 350 nm ($R^2 = 0.94$, $F_{1,17} = 235.63$, $p < 0.001$, $y = 2.273e + 10x - 2.805e + 10$; Fig. 1). Additionally, significant positive relationships were detected at 400 ($R^2 = 0.93$, $F_{1,17} = 231.31$, $p < 0.001$, $y = 2.358e + 10x -$

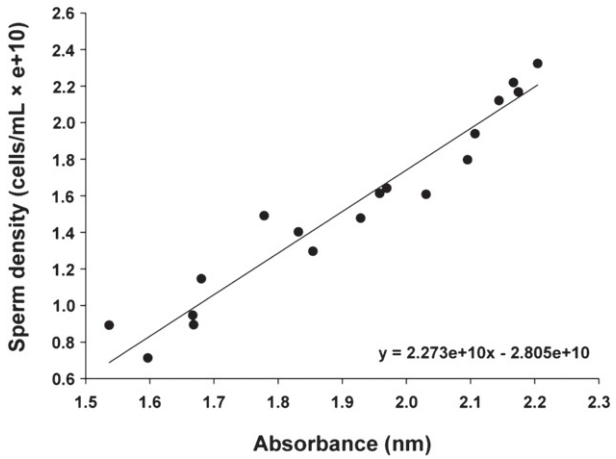

Fig. 1. Relationship between sperm density and absorbance for the European eel, *Anguilla anguilla*. Density was determined using a haemocytometer and absorbance was determined at 350 nm by use of a spectrophotometer.

2.745e + 10), 500 ($R^2 = 0.93$, $F_{1,17} = 227.36$, $p < 0.001$, $y = 2.476e + 10x - 2.628e + 10$), and 600 nm ($R^2 = 0.93$, $F_{1,17} = 220.50$, $p < 0.001$, $y = 2.472e + 10x - 2.408e + 10$).

3.2. Experiment 2: Determination of the optimal sperm to egg ratio

Mean fertilization success, using sperm to egg ratios ranging from 1.3e + 3 to 1.0e + 6 sperm per egg increased from 37.5 to 68.1%, respectively (Fig. 2). Sperm to egg ratio had a significant effect on fertilization success ($F_{11,180} = 12.24$; $p < 0.0001$; Fig. 2). Fertilization success increased from 1.3e + 3 to 2.5e + 4 sperm per egg, while adding greater than 2.5e + 4 sperm per egg had no significant effect on fertilization success (Fig. 2). The female VC was significant for fertilization success ($p < 0.0001$) and explained 86.4% of the models variance, while the residual error explained only 13.6%.

3.3. Experiment 3: Effect of time post-stripping

Duration of time post-stripping had a significant effect on fertilization success ($F_{7,50.1} = 9.94$; $p < 0.0001$; Fig. 3). Between 0 and 10 min post-stripping 57.4 to 78.2% of the eggs were fertilized on average (Fig. 3). At 15 min post-stripping, a significant decrease in fertilization success was detected; i.e. decreased to 35.2% at 60 min post-stripping. The female VC was significant for fertilization success ($p < 0.0001$) and explained 84.3% of the models variance while the residual error explained 15.7%.

4. Discussion

In this study, we report several key findings: (i) haemocytometer counts for quantification of sperm density were positively related to absorbance by use of a spectrophotometer, (ii) sperm to egg ratio had a significant effect on fertilization success, where adding greater than 2.5e + 4 sperm per egg had no significant effect, (iii) duration of time post-stripping had a significant effect on egg fertilization success, such that at 15 min post-stripping a significant decrease in fertilization success was detected, and (iv) the female VC was significant for fertilization success (for both Experiments) and explained ≥ 84% of the models variance.

Here, we showed that European eel sperm could be rapidly and accurately (94% of the variance was explained) quantified by use of a spectrophotometer. Similar relationships have also been established for small-bodied biomedical fishes (Tan et al., 2010), as well as economically important marine (Rouxel et al., 2008), and freshwater species (Ciereszko and Dabrowski, 1993). To establish our method, we tested four different wavelengths spanning the visible spectrum. Our results showed highly significant relationships among readings at 350 to 600 nm, indicating that all tested wavelengths can be used for quantifying sperm density for this species. Using small-bodied biomedical model fishes, Tan et al. (2010) observed a similar phenomenon; i.e. no single maximal absorbance peak was detected across the visible spectrum (380 to 750 nm). This likely occurs because of variability in milt composition, through an assortment of lipids and proteins (among others), which essentially makes it difficult to find a discrete peak in the absorbance spectra via spectrophotometry (Dong et al., 2005). Nevertheless, the 350 nm wavelength appeared to be the most suitable for sperm quantification based on having a slightly higher coefficient of determination ($R^2 = 0.94$ vs. 0.93). Ultimately, this methodology can now be incorporated into routine hatchery and experimental protocols to avoid potential confounding factors relating to male-to-male variation in sperm density. Additionally, it should increase the effectiveness of cryopreservation and fertilization protocols by standardizing the number of cells in each cryogenic sperm straw and optimizing sperm to egg ratios (Alavi et al., 2008; Butts et al., 2011; Ciereszko et al., 2000), respectively.

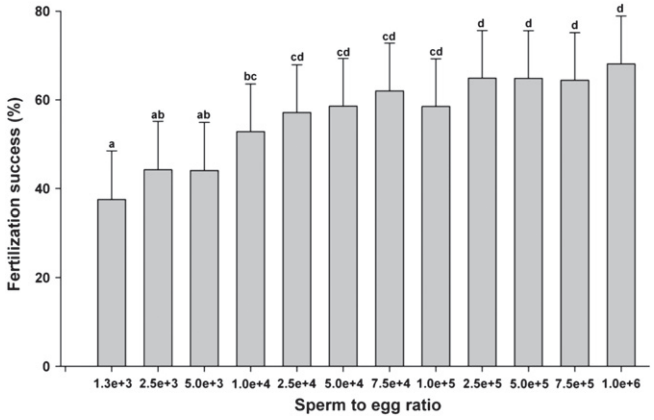

Fig. 2. The effect of sperm to egg ratio on fertilization success in the European eel, *Anguilla anguilla*. Sperm to egg ratios with different letters are significantly different ($p < 0.05$, least square means, ANOVA). Error bars represent least square means standard error.

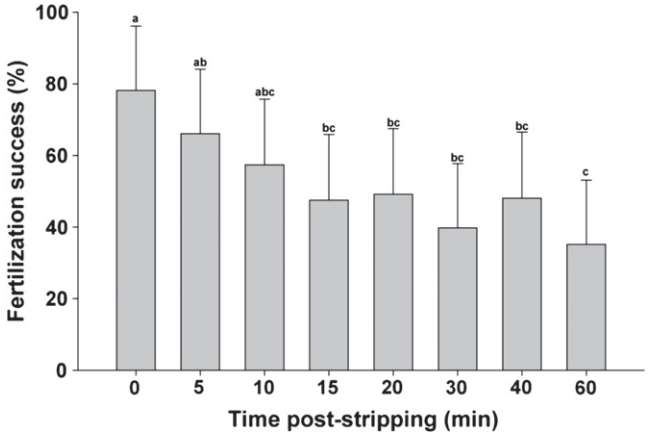

Fig. 3. The effect of time post-stripping of eggs on fertilization success in the European eel, *Anguilla anguilla*. Times with different letters are significantly different ($p < 0.05$, least square means, ANOVA). Error bars represent least square means standard error.

Based on a literature review by Butts et al. (2012a), the number of sperm required to fertilize a fish egg is relatively high and species dependent (mean ± SEM for 15 species was ~550,000 ± 480,000 sperm per egg). To our knowledge, this is the first study that assesses how sperm number influences egg fertilization for the European eel. An increase in fertilization success was observed up to 2.5e + 4 sperm per egg; adding additional sperm had no significant effect on the dependent variable. Consequently, this gamete ratio is now recommended for studies dealing with European eel fertility, as it secured maximal fertilization rates, while at the same time used limited sperm cells. However, when using this ratio we still have to be cognizant of the fertilization environment, as varying the sperm extender, adding additional activation media, "crossing" gametes in containers with different dimensions, and modifying gamete contact times may all influence the final outcome of a fertilization event, thus altering the optimal sperm to egg ratio (Alavi et al., 2008; Casselman et al., 2006; Ciereszko et al., 2000). Thus, these variables (among other factors) should coincide with those used in our experimentation, especially if our optimized sperm to egg ratio is to be used for fertilization. Ultimately, this will enable the fertilizing capability of individual males to be measured consistently and each male's sperm, whether stored frozen or in living gene banks, to be used wisely.

We found that at 15 min post-stripping of the eggs, a significant decrease in fertilization success was detected. This suggests that European eel eggs are much more sensitive to storage time post-stripping relative to that of salmonids, such as Chinook- (*Oncorhynchus tshawytscha*) and Atlantic salmon (*Salmo salar*), where a delay in activation did not affect fertilization for these species until 1 h post-stripping (Munkittrick et al.,

1992). In the Japanese eel (*Anguilla japonica*), Ohta et al. (1996) found that time post-ovulation, within the ovary, to in vitro egg activation is an important factor for fertilization with a clear negative effect occurring at 6 to 7 h post-ovulation. This suggests that we should mimic this ovarian environment in situ to decrease this rapid rate of egg decay as observed in our study; perhaps through extenders that mimic the ovarian fluid of European eel under a strict thermal and/or oxygenated regime.

Fertilization success was strongly influenced by female identity. In our studies, eggs were obtained from wild-caught females that experienced different conditions prior to capture and size/age varied, although they were housed under similar conditions during the reproductive season (e.g. photoperiod, temperature) and received similar hormonal therapy. Ultimately, these factors may have contributed to this variability in female responsiveness to treatment and egg quality. Eggs from each female were fertilized using a new sperm pool from multiple males (having no difference in sperm motility), thus masking any potential paternal effects that may have been active during and immediately after embryogenesis (Rideout et al., 2004). Nevertheless, there is still a suite of other genetic and environmental factors, as well as the associated genetic × environmental interaction, which modulate nuclear-genetic and extra-nuclear non-genetic constituents (e.g. yolk, lipids, immunoglobulin, mRNAs, hormones, and stage of oocyte maturation) that foster the development, quality, and size of an egg, in turn influencing female fertility (reviewed in Babin et al., 2007). Future studies should explore these maternal processes, to ultimately decipher the direct mechanisms leading to enhanced egg quality and higher fertilizations success for this species.

In conclusion, we have now standardized artificial fertilization protocols for the European eel under controlled experimental conditions. Based on our findings, we conclude that spectrophotometry is a valuable tool for estimating sperm density for this species. Furthermore, we suggest that European eel eggs should be fertilized using 2.5e + 4 sperm per egg within the first 10 min post-stripping to maintain a high capacity to be fertilized. Overall, these findings will contribute to the development of European eel breeding technology and further our understanding on sperm biology, cryobiology, and reproductive biology in fishes.

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References

Alavi, S.M.H., Linhart, O., Coward, K., Rodina, M., 2008. Fish spermatology: implications for aquaculture management. In: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Ltd., Oxford, pp. 397–461.

Babin, P.J., Cerdà, J., Lubzens, E., 2007. The fish oocyte: from basic studies to biotechnological applications. Springer, New York.

Ball, M.A., Parker, G.A., 1996. Sperm competition games: external fertilization and "adaptive" infertility. J. Theor. Biol. 180 (2), 141–150.

Bergh, Ø., Hansen, G.H., Taxt, R.E., 1992. Experimental infection of eggs and yolk sac larvae of halibut, *Hippoglossus hippoglossus* L. J. Fish Dis. 15 (5), 379–391.

Bohonak, A.J., 2004. RMA: software for reduced major axis regression, version 1.17. San Diego State University, San Diego.

Butts, I.A.E., Trippel, E.A., Litvak, M.K., 2009. The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod *Gadus morhua* L. Aquaculture 286 (1–2), 89–94.

Butts, I.A.E., Babiak, I., Ciereszko, A., Litvak, M.K., Słowińska, M., Soler, C., Trippel, E.A., 2011. Semen characteristics and their ability to predict sperm cryopreservation potential of Atlantic cod, *Gadus morhua* L. Theriogenology 75 (7), 1290–1300.

Butts, I.A.E., Roustaian, P., Litvak, M.K., 2012a. Fertilization strategies for winter flounder: effects of spermatozoa density and the duration of gamete receptivity. Aquat. Biol. 16 (2), 115–124.

Butts, I.A.E., Love, O.P., Farwell, M., Pitcher, T.E., 2012b. Primary and secondary sexual characters in alternative reproductive tactics of Chinook salmon: associations with androgens and the maturation-inducing steroid. Gen. Comp. Endocrinol. 175 (3), 449–456.

Casselman, S.J., Schulte-Hostedde, A.I., Montgomerie, R., 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can. J. Fish. Aquat. Sci. 63 (9), 2119–2125.

Ciereszko, A., Dabrowski, K., 1993. Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. Aquaculture 109 (3–4), 367–373.

Ciereszko, A., Glogowski, J., Dabrowski, K., 2000. Fertilization in landlocked sea lamprey: storage of gametes, optimal sperm:egg ratio, and methods of assessing fertilization success. J. Fish Biol. 56 (3), 495–505.

Dollerup, J., Graver, C.M., 1985. Repeated induction of testicular maturation and spermiation, alternating with periods of feeding and growth in silver eels, *Anguilla anguilla* (L.). Dana J. Fish. Mar. Res. 4, 19–39.

Dong, Q.X., Eudeline, B., Huang, C., Tiersch, T.R., 2005. Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg). Aquac. Res. 36 (1), 86–93.

Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L., Asturiano, J.F., 2012. Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354, 7–16.

Gousset, B., 1990. European eel (*Anguilla anguilla* L.) farming technologies in Europe and in Japan: application of a comparative analysis. Aquaculture 87 (3–4), 209–235.

Hatef, A., Niksirat, H., Alavi, S.M.H., 2009. Composition of ovarian fluid in endangered Caspian brown trout, *Salmo trutta caspius*, and its effects on spermatozoa motility and fertilizing ability compared to freshwater and a saline medium. Fish Physiol. Biochem. 35 (4), 695–700.

Johnston, T.A., Kaufman, S.D., Moles, M.D., Wiegand, M.D., Leggett, W.C., 2008. Effects of delayed fertilization on embryo viability in walleye *Sander vitreus* (Mitchill): the role of maternal effects. J. Fish Biol. 72 (10), 2634–2644.

Kagawa, H., Tanaka, H., Ohta, H., Unuma, T., Nomura, K., 2005. The first success of glass eel production in the world: basic biology on fish reproduction advances new applied technology in aquaculture. Fish Physiol. Biochem. 31 (2–3), 193–199.

Ludbrook, J., 2010. Linear regression analysis for comparing two measurers or methods of measurement: but which regression? Clin. Exp. Pharmacol. Physiol. 37 (7), 692–699.

Mordenti, O., Di Biase, A., Bastone, G., Sirri, R., Zaccaroni, A., Parmeggiani, A., 2013. Controlled reproduction in the wild European eel (*Anguilla anguilla*): two populations compared. Aquac. Int. 21 (5), 1045–1063.

Munkittrick, K.R., McGeachy, S.M., Burke, M.G., Flett, P.A., 1992. Effects of delay in water addition or rinsing on fertilization rates of Chinook salmon, coho salmon, Atlantic salmon, and rainbow trout eggs. Progress. Fish Cult. 54 (1), 14–20.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K., 1996. Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica*. Aquaculture 139 (3–4), 291–301.

Ottolenghi, F., Silvestri, C., Giordano, P., Lovatelli, A., New, M.B., 2004. Eels. Capture-based aquaculture — the fattening of eels, groupers, tunas and yellowtails. FAO, Rome, Italy.

Palstra, A.P., Cohen, E.G.H., Niemantsverdriet, P.R.W., van Ginneken, V.J.T., van den Thillart, G.E.E.J.M., 2005. Artificial maturation and reproduction of European silver eel: development of oocytes during final maturation. Aquaculture 249 (1–4), 533–547.

Peñaranda, D.S., Pérez, L., Gallego, V., Barrera, R., Jover, M., Asturiano, J.F., 2010. European eel sperm diluent for short-term storage. Reprod. Domest. Anim. 45 (3), 407–415.

Pérez, L., Peñaranda, D., Gallego, V., Asturiano, J., 2009. Testis development, sperm quality evaluation and cryopreservation in the European eel. In: Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning Migration of the European Eel, vol. 30. Springer, Dordrecht, pp. 333–362 (Netherlands).

Psenicka, M., Rodina, M., Linhart, O., 2010. Ultrastructural study on the fertilisation process in sturgeon (*Acipenser*), function of acrosome and prevention of polyspermy. Anim. Reprod. Sci. 117 (1–2), 147–154.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. Paternal effects on haddock early life history traits. J. Fish Biol. 64 (3), 695–701.

Rouxel, C., Suquet, M., Cosson, J., Severe, A., Quemener, L., Fauvel, C., 2008. Changes in Atlantic cod (*Gadus morhua* L.) sperm quality during the spawning season. Aquac. Res. 39 (4), 434–440.

SAS Institute Inc., 2003. SAS System, version 9.1. SAS Institute Inc., Cary, NC, USA.

Snook, R.R., Hosken, D.J., Karr, T.L., 2011. The biology and evolution of polyspermy: insights from cellular and functional studies of sperm and centrosomal behavior in the fertilized egg. Reproduction 142 (6), 779–792.

Sørensen, S.R., Gallego, V., Pérez, L., Butts, I.A.E., Tomkiewicz, J., Asturiano, J.F., 2013. Evaluation of methods to determine sperm density for the European eel, *Anguilla anguilla*. Reprod. Domest. Anim. 48 (6), 936–944.

Spilke, J., Piepho, H.P., Hu, X., 2005. Analysis of unbalanced data by mixed linear models using the mixed procedure of the SAS system. J. Agron. Crop Sci. 191 (1), 47–54.

Støttrup, J.G., Jacobsen, C., Tomkiewicz, J., Jarlbaek, H., 2013. Modification of essential fatty acid composition in broodstock of cultured European eel *Anguilla anguilla* L. Aquac. Nutr. 19 (2), 172–185.

Tan, E., Yang, H., Tiersch, T., 2010. Determination of sperm concentration for small-bodied biomedical fishes by use of microspectrophotometry. Zebrafish 7 (2), 233–240.

Tomkiewicz, J., Tybjerg, L., Støttrup, J., McEvoy, F., Ravn, P., Sørensen, S.R., Lauesen, P., Graver, C., Munk, P., Holst, L.K., Vestbö, B., Svalastoga, E., Jacobsen, C., Holst, B., Steenfeldt, S.J., Buelund, L., Hornum, T., Kofoed, T., 2012. Reproduction of European Eel in Aquaculture (REEL). DTU Aqua Report Series (47 pp.).

Zar, J.H., 1996. Biostatistical analysis, Third edition. Prentice-Hall, New Jersey.