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Abstract

During European eel assisted reproduction, timely administration of hormones that induce oocyte maturation and ovulation is a major factor influencing subsequent egg quality. This treatment commonly comprises one injection of fish pituitary extract (PE) as a primer followed by a 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) injection. In this context, the present study aimed at optimizing timing of the dual hormone administration by applying a lipid droplet-based oocyte maturation scale, previously developed for Japanese eel to determine the maturational status of each female. Using wild-caught female eels, the potential effect of female size, egg fatty acid composition and dry weight on egg quality was also analyzed. Larval survival at 3 days post hatch was used to differentiate High- and Low-quality egg batches. Results showed that lipid droplet diameter was significantly smaller in High-quality eggs than in Low-quality egg batches, indicating that females producing High-quality eggs received the PE primer and DHP generally at an earlier developmental stage than those producing Low-quality batches. These results confirm that oocyte lipid droplet diameter is a useful indicator of female maturational status for optimization of induction of oocyte maturation and ovulation in European eel. Additional parameters, including female size, egg fatty acid composition and dry weight, were similar between high and low quality egg batches. This insight regarding the fatty acid composition of eggs obtained from wild-caught female eels may help advancing the development of tailored diets for increased reproductive success of farmed broodstock.

Keywords	European eel, assisted reproduction, lipid droplets, oocyte maturation, fatty acids, egg quality
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Highlights:

- Oocyte lipid droplet diameter at induction of maturation and ovulation influenced resulting egg quality.
- Hatching success and larval viability was enhanced by induction at an earlier stage of oocyte maturation.
- Egg fatty acid composition was similar between egg batches of high and low quality.

1 Oocyte and egg quality indicators in European eel: lipid droplet coalescence and fatty acid composition

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Abstract

During European eel assisted reproduction, timely administration of hormones that induce oocyte maturation and ovulation is a major factor influencing subsequent egg quality. This treatment commonly comprises one injection of fish pituitary extract (PE) as a primer followed by a 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) injection. In this context, the present study aimed at optimizing timing of the dual hormone administration by applying a lipid droplet-based oocyte maturation scale, previously developed for Japanese eel to determine the maturational status of each female. Using wild-caught female eels, the potential effect of female size, egg fatty acid composition and dry weight on egg quality was also analyzed. Larval survival at 3 days post hatch was used to differentiate High- and Low-quality egg batches. Results showed that lipid droplet diameter was significantly smaller in High-quality eggs than in Low-quality egg batches, indicating that females producing High-quality eggs received the PE primer and DHP generally at an earlier developmental stage than those producing Low-quality batches. These results confirm that oocyte lipid droplet diameter is a useful indicator of female maturational status for optimization of induction of oocyte maturation and ovulation in European eel. Additional parameters, including female size, egg fatty acid composition and dry weight, were similar between high and low quality egg batches. This insight regarding the fatty acid composition of eggs obtained from wild-caught female eels may help advancing the development of tailored diets for increased reproductive success of farmed broodstock.

Key words: European eel, assisted reproduction, lipid droplets, oocyte maturation, fatty acids, egg quality

1. Introduction

European eel (*Anguilla anguilla*) aquaculture presently relies on wild-caught juveniles. Therefore, the development of hatchery technology for aquaculture production is in progress with focus on producing large quantities of viable offspring. However, sexual maturation in eel does not occur spontaneously in captivity due to a strong dopaminergic inhibition (Dufour et al., 1988; Vidal et al., 2004) and low gonadotropin synthesis and release (Dufour et al., 1983). Gonadal development is therefore commonly induced using exogenous hormones adopting a protocol described by Ohta et al. (1996) for Japanese eel with some adaptations to European eel. Typically, hormonal treatments consist of repeated weekly

injections of salmon or carp pituitary extracts (SPE or CPE) for females and human chorionic gonadotropin in males. Additionally, in female eels, oocyte maturation and ovulation is induced applying a priming dose of PE followed by provision of a maturation-inducing hormone (MIH), commonly 17α, 20β-dihydroxy-4-pregnen-3-one (DHP). Despite significant progress in assisted reproduction techniques (Butts et al., 2014; Di Biase et al., 2017; Mordenti et al., 2013; Palstra et al., 2005; Pedersen, 2003, 2004; Tomkiewicz, 2012), sub-optimal final maturation treatment of female eels frequently lead to unsuccessful ovulation, challenging the successful production of viable eggs.

Application of biomarkers as indicators of egg viability is a useful tool in aquaculture to optimize resources and to better understand the underlying mechanisms that determine high egg and larvae quality. In case of the Anguillid eels, the maturational status and developmental capacity of follicles in the ovary at induction of maturation and ovulation is an important factor determining the subsequent egg quality. To identify the timing for inducing oocyte maturation and ovulation, a common practice is to assess ovarian maturational status through the female weight increase followed by ovarian biopsies to assess oocyte maturational stage. In Japanese eel (*Anguilla japonica*), oocyte maturation and ovulation have been induced successfully based on body weight increase and oocyte diameter (Kagawa et al., 1995; Ohta et al., 1996). However, the same protocol applied to European female eels resulted in low reproductive success, with a few hatched larvae, embryonic malformation and high mortality (Pedersen, 2003, 2004). In species with ooplasm lipid formation, like the eel, a common feature of oocyte cytoplasmic maturation is the coalescence of numerous small lipid droplets that after ovulation form one or a few large lipid droplets (Kagawa et al, 2013; Lubzens et al., 2010, 2017). These cytological changes may serve as biomarkers for assessment of oocyte maturational status in relation to assisted reproduction procedures. For Japanese eel, a lipid droplet-based oocyte scale has been developed and proven useful as a quantitative measure to evaluate oocyte maturational status and determine the optimal timing of induction of oocyte maturation and ovulation (Unuma, et al., 2011). In European eel, the determination of oocyte developmental stage is presently based on a seven-stage scale developed by Palstra et al. (2005), which focuses on the position of the migratory germinal vesicle, lipid droplet size and general appearance of the oocytes. However, high variability on the appearance of developing follicles and the lack of correlation with subsequent egg viability calls for validation of this methodology.

Another common factor influencing egg quality in fish is the nutritional status of the female, affecting among other, egg dry weight, total lipid and fatty acid composition (Izquierdo et al., 2001). In European eel, egg production and egg viability is generally higher in wild-caught female silver eels compared to cultured female eels (Tomkiewicz, 2012). By experiencing a natural feeding regime and naturally starting the spawning migration, wild-caught female silver eels should be capable to produce eggs containing the nutritional requirements for normal embryonic and yolk-sac larval development and survival. Therefore, differences in egg quality between wild and cultured broodstock may arise due to differences in female pre-spawning nutrition, in particular fatty acid composition, as it has been observed in e.g. black sea bass (*Centropristis striata*) (Seaborn et al., 2009) and Atlantic cod (*Gadus morhua*) (Lanes et al., 2012). In European eel, the effect of maternal dietary fatty acid composition on egg composition and quality has shown for farmed eels on different diets (Støttrup et al., 2013, 2016). Moreover, the fatty acid composition of broodstock diet and essential fatty acid content significantly influence ovarian development (da Silva et al., 2016) and egg quality in both Japanese and European eel (Furuita et al., 2003, 2006; Heinsbroek et al., 2013; Støttrup et al., 2016). Investigating the fatty acid composition of eggs from wild-caught female eels can add new and valuable information contributing to improve the reproductive success of farmed broodstock and assessment of egg quality validated through larval viability.

The aim of this study was to identify and evaluate egg quality indicators in European eel with focus on oocyte characteristics at the time of hormonal administration while considering influences of female weight and length as well as egg dry weight and fatty acid composition. As indicators of egg quality, we determined fertilization and hatching success, larval survival and larval longevity. Larval survival at 3 days post hatch (dph) was used as quality criterion for egg quality classification. This is an early estimate of larval viability reflecting the influence of hormonal treatments, while the effects due to rearing conditions are minimized (Kjørsvik et al., 1990).

2. Material and Methods

2.1 Ethics statement

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental breeding protocols were approved by the

Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2010/561-1783). All efforts were made to minimize animal handling and stress.

2.2 Broodstock rearing conditions

European female silver eels (n=16, body weight 748 ± 362 g; body length 71 ± 10 cm), were caught in the autumn of 2012 in a freshwater lake (Vandet Sø) in northern Jutland (Denmark) and transported to a research facility of the Technical University of Denmark located at Lyksvad Fish Farm (Vamdrup, Denmark). Eels were randomly distributed into 300 l tanks equipped with a recirculation system and were gradually acclimatized to salt water over a two week period, i.e. fresh water adjusted artificially to 36 psu salinity using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany). Male eels (n=30, body weight 106 ± 13 g, body length 38 ± 2 cm), reared on DAN-EX 2848 (BioMar A/S, Brande, Denmark) were obtained at a commercial eel farm (Stensgård Eel Farm A/S, Randbøl, Denmark), transported to Lyksvad Fish Farm and kept in separate tanks under the same conditions as the female eels. At the onset of hormonal treatments, each fish was anaesthetized in an aqueous solution of benzocaine (ethyl p-aminobenzoate, 20 mg/l, Sigma-Aldrich, Germany), tagged with a passive integrated transponder (PIT tag) and body weight and length were measured. Throughout the experiment, all fish were maintained at ~36 psu (salinity), ~20 °C, and a natural local daily photoperiod was used. No feed was provided during experiments since eels in the migratory stage naturally cease feeding (Lokman et al., 2003).

2.3 Induction of gametogenesis and gamete extraction

Oogenesis was induced by weekly intramuscular injection of salmon pituitary extract (SPE) at a constant dosage of 18.75 mg/kg initial body weight (Kagawa et al., 2005; Ohta et al., 1996; Tomkiewicz et al., 2012). Pituitary extract was prepared using freeze-dried salmon pituitaries (Argent Chemical Laboratories, Washington, USA) that were grinded, diluted in NaCl 0.9 g/l and centrifuged (Ohta et al., 1996, 1997). Supernatants were stored at -20 °C until use. Females were weighed and inspected at the weekly injections to follow changes in body weight. At the onset of treatment, the females were weighed, PE dose determined and the first injection given concurrent with the tagging. Subsequently, each female was weighed and PE-treated weekly over a period of 16-20 weeks, depending on the female responsiveness. Induction of follicular maturation and ovulation was adapted

to each female and initiated when the body weight at the weekly injection showed an increase of 10-15 % compared to the initial body weight in combination with an increasing softness of the abdomen (Pedersen, 2003, 2004). Thereafter the female was transferred to a separate 300 l tank under the same conditions for individual care and the body weight observed the following day. In case of continued weight increase, oocyte stage was assessed, i.e., the female was anaesthetized in an aqueous solution of benzocaine, and an ovarian biopsy (~0.2 ml) was obtained, using a sterile disposable injection needle (16G x 1 ½"). The biopsy was taken at a standard location on the left side of the body, 5-10 cm anterior to the genital pore. The biopsy was inspected under the microscope and oocyte development graded on a scale from 1-7 according to Palstra et al. (2005). Progression of oocyte maturation varied in time and homogeneity, so each female was followed until the most developed oocytes exhibited characteristics of stage 4, i.e., transparent oocyte with a peripheral germinal vesicle. At this stage, an additional SPE injection as primer was given to females to boost follicular development (Kagawa et al., 2005; Pedersen, 2004). The timing of the primer treatment varied from 1-6 days after the standard weekly injection.

To complete follicular maturation and induce ovulation, the eel maturation inducing steroid, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma-Aldrich Chemie, Steinheim, Germany) was given ~24 h later at a dose of 2 mg/kg present body weight) (Ohta et al., 1996). Prior to DHP injection, a new biopsy (~0.2 ml) was obtained to evaluate the progression of oocyte development. DHP was injected into the ovarian tissue under anesthesia (Palstra et al. 2005). Ovulation occurred around ~14 hours after DHP injection and eggs were stripped by applying gentle pressure along the abdomen of the fish. The weight of ovulated eggs collected was recorded. After stripping, females in good condition that re-gained body weight received the same treatment for oocyte maturation and ovulation a second time (Table 1).

The treatment to induce spermatogenesis in the male eels started four weeks later than the female one as male maturation need shorter time (7-9 weekly injections to reach spermiation). Spermatogenesis was induced by weekly injections of human chorionic gonadotropin (hCG) at 1.5 IU/g (Sigma Aldrich Denmark, A/S) (Pérez et al., 2000) until the end of the experiment. Once, the males matured, they were capable of providing milt for over the entire experimental period. For each egg batch, milt was collected from three to four males 2 hours prior to expected fertilization by applying a gentle pressure

to the abdominal area. The pooled milt was diluted in sterile filtered artificial seminal plasma medium (Asturiano et al., 2004). As the sperm concentration in the milt varies, the spermatocrit was used to standardize the volume of milt used in the dilution (Sørensen et al., 2013). Thus, a pooled milt sample was spun for 10 min at 6000 × g (Haematokrit 210, Andreas Hettich GmbH & Co.KG, Tuttlingen Germany) to assess the spermatocrit and the dilution was adjusted to a sperm concentration of 1:99 (Ohta et al., 1996). The sperm motility was assessed within 30 seconds of activation using a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan), equipped with a Nikon 400 × magnification (40 × CFI Plan Flour). An arbitrary scale, i.e. 0: no motile sperm; I: <25 %; II: 25-50 %; III: 50-75 %; IV: 75-90 %; and V: 90-100 % was used to range the proportion of motile spermatozoa (Sørensen et al., 2013). Only milt with motility higher that than 75 % was applied. Diluted milt was kept in sterile culture flasks at 20˚C prior to fertilization (within 2 hours post-stripping).

2.4 Fertilization, embryonic and larval survival

Stripped eggs from each female were fertilized with the pre-diluted milt using a ratio of 1 ml of diluted milt per 2 g of newly stripped eggs according to Sørensen et al. (2016). Accordingly, gamete activation was initiated by seawater 32.5 psu filtered 0.2 micron (CUNO 3M®, St. Paul, MN, USA) and adjusted to targeted salinity (± 0.1 psu) using Tropic Marin Sea Salt (Dr. Biener Aquarientechnik, Wartenberg, Germany) and an electronic conductivity meter (WTW Multi 3410 + TetraCon325, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Salinity of seawater for activation was adjusted taking into consideration the salinity of P1 milt diluent being 10.3 psu and the salinity during fertilization being 36 psu.

After 5 min gamete contact time, eggs were transferred to 10 l containers for separation of floating and sinking eggs. The eggs were kept at 20 °C. At 3-5 hpf, a sample of 100-150 floating eggs was collected estimate fertilization success. Eggs were photographed using an optical microscope (Eclipse 55i, Nikon Corporation, Japan) at 20X magnification and a digital camera (Digital Sight DS-Fi1, Nikon Corporation, Japan). Fertilized eggs were identified by the presence of blastomere cleavage (4 to 64 cell stage), while those that had not reached the 4-cellstage were considered unfertilized (Sørensen et al., 2016).

For estimation of embryonic survival and hatching success, ~200 eggs in triplicate for each female were collected from the floating layer after 3-5 hpf and incubated at 20 °C in flasks (Nunc® 75 cm^2

186 flasks, non-treated with ventilated caps, Thermo Scientific) (Sørensen et al. 2014). Each flask
187 contained 250 ml of seawater (36 psu), ampicillin (50 mg/l) and rifampicin (50 mg/l). Flasks stayed
188 undisturbed inside a dark and closed incubator at 20 °C until the number of dead and live embryos was
189 counted at 30 and 40 hpf (embryonic survival) and the number of dead and hatched larvae at 55 hpf
190 (hatching success). To estimate larval survival ~300 g of eggs from the floating layer were incubated in
191 a 60 l incubator with seawater (adjusted to 36 psu salinity using Tropic Marine Sea Salt) at ~20 °C.
192 After hatch, when available ~3000 larvae in triplicate were stocked in 40 l tanks of an recirculation
193 aquaculture system (RAS) containing seawater adjusted to 36 psu salinity using Tropic Marine Sea Salt
194 and reared system at 20 °C. Larval survival, i.e. the percentage of living larvae, was estimated daily.
195 Longevity, i.e. the number of days post hatch (dph) that larvae survived, was also recorded for each
196 batch. Larval survival at 3 dph was used to classify egg quality groups. When data on larval survival at
197 3 dph was not available (female D593 and DDF2), longevity was used to assess egg quality.

198 2.5 Sampling, lipid droplet diameter and oocyte stage

199 During the induction of oocyte maturation and ovulation, the following samples were collected: 1) ~0.2
200 ml of ovarian biopsy obtained ~1 hour before the SPE priming injection (SPE); 2) ~0.2 ml of ovarian
201 biopsy obtained ~1 hour prior to DHP injection (DHP); 3) a sample of unfertilized eggs collected
202 immediately after stripping (EGG) and 4) a sample of fertilized eggs collected 5 hours post fertilization
203 (hpf). Each sample was micro-photographed and used for measurements of oocyte lipid droplet
204 diameter. Images were taken using a digital camera (Digital Sight DS-Fi1, Nikon Corporation)
205 connected to an objective microscope (Eclipse 55i, Nikon Corporation, Japan) at 2× magnification.

206 Lipid droplet diameter was measured according to Unuma et al. (2011). In short, using the digital
207 images of each sample (SPE, DHP, EGG and 5 hpf), ten oocytes were randomly selected among those
208 at the most advanced stage of development. In each oocyte, ten of the largest lipid droplets were
209 measured and the maximum five values averaged. For each lipid droplet, the diameter was calculated
210 by the average of two diameter measurements. At the final stages of coalescence, only a few droplets
211 became larger while the others became smaller, in these stages, the diameter was based on the diameter
212 of the largest droplet only. Oocyte stage was attributed to each oocyte according to the criteria used in
213 the oocyte scale developed for Japanese eel (Unuma et al., 2011), where in stage 1: the width of the
214 transparent zone is less than 25 % of the oocyte diameter; in stage 2: the average diameter of the five

largest lipid droplets is under 40 μm and the width of the transparent zone is more than 25 % of the oocyte diameter; in stage 3: the average diameter of the five largest lipid droplets is from 40 to 55 μm; in stage 4: the average diameter of the five largest lipid droplets is from 55 to 70 μm; in stage 5: the average diameter of the five largest lipid droplets is from 70 to 90 μm; in stage 6: the average diameter of the five largest lipid droplets is from 90 to 110 μm; in stage 7: the average diameter of the five largest lipid droplets is from 110 to 130 μm; in stage 8: the diameter of the largest lipid droplet is less than 190 μm. The average diameter of the five largest lipid droplets is more than 130 μm; in stage 9: the diameter of the largest lipid droplet is from 190 to 250 μm; in stage 10: the diameter of the largest lipid droplet is more than 250 μm. The median stage of the oocytes analyzed was defined as the stage for the individual. Lipid droplet diameter was measured using the free software ImageJ 1.48d.

2.6 Estimation of egg dry weight

For determination of egg dry weight, a sample of unfertilized eggs (~0.1 ml) from each batch was transferred to pre-weighed plastic weighing boats and weighed. The weighing boats and eggs were then dried at 60 °C for 24 h (or until the weight was stable) and weighed again. For each batch, the slope of the regression line between wet weight and the corresponding number of eggs in the sample was calculated by weighting 4 samples of 0.1 ml of eggs from each batch to another pre-weighed weigh boat. These eggs were then transferred to a 1.5 ml Eppendorf tubes containing 4% formalin (Hounisen Laboratory Equipment A/S, Risskov, Denmark) for later counting. In order to calculate the number of eggs in each dried egg sample, the value of the regression slope was multiplied by the weight of the sample before drying (wet weight). For each batch, the mean dry weight per egg was then calculated as dry weight of the sample divided by the number of eggs calculated for that sample. All weighing and drying steps were done in triplicate for each egg batch.

2.7 Fatty acid analysis

Lipid extraction and content

Lipid in eggs (4-6 g samples) were extracted with a homogeneous mixture of chloroform, methanol and water (2:2:1.8) following the method of Bligh and Dyer (1959). The lipid extracts were frozen at -40 °C for the subsequent determination of lipid content, lipid class fractionation and fatty acid composition. The lipid content was determined by gravimetry after evaporation of chloroform.

Lipid class separation

Lipids from egg extracts were separated into polar (PL) and neutral lipids (NL) by chromatography on a solid phase consisting of aminopropyl-modified silica. Solvents with increasing polarity were used to separate the lipid classes. A lipid extract corresponding to 10–100 mg lipid was used for the lipid class separation. Solvents from the lipid extraction were evaporated, and the extract was re-solubilized in 0.5 ml chloroform and transferred to a Sep- Pak column (Waters Corporation, Milford, MA, USA). NL were eluted using 4 ml chloroform/2-pronanol (2:1), and PL were eluted with 6 ml methanol. The PL fraction includes both phospholipids and glycolipids.

Fatty acid methyl esters and fatty acid composition

Eluates from lipid class separation of egg fat extracts were used for the preparation of fatty acid methyl esters, applying AOCS Official Method Ce 2-66 (Anon 1998). C23:0 methylester was used as internal standard. Fatty acid methyl esters were analyzed on a HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA). Fatty acids were identified by comparison of retention times with a mixture of standards containing all the fatty acids identified in this study. Each fatty acid was quantified by calculating its peak area relative to the total peak area. Hereafter, these values are referred to as fatty acid content (% weight of total fatty acids).

2.8 Statistical analysis

For statistical analysis, egg batches were categorized into two groups: i) High-quality and ii) Low-quality. Survival beyond 3 dph was used to differentiate egg quality groups (Table 1). All data were analyzed using R version 3.1.3 (R core team, 2015). Residuals were tested for normality (Shapiro-Wilk test) and homogeneity of variance (plot of residuals versus predicted values). Linear regression analysis was used to determine whether there were significant correlations between fertilization success and embryonic survival estimated at 30, 40 hpf and hatching success estimated at 55 hpf. Linear regression analysis was also used to determine possible relations between fatty acid composition (in both NL and PL) and fertilization success, embryonic survival and hatching success. Pearson correlation coefficient (R) was calculated to measure the strength of these linear relationships. Embryonic survival, fertilization and hatching success were not normally distributed. Therefore, for these parameters, a series of non-parametric Wilcoxon signed-rank tests were used to test possible differences between Low- and High-quality groups. Welch's t-test was also used to test differences in fatty acid levels and

lipid droplet size (at SPE, DHP, EGG and 5 hpf) between the Low- and High-quality egg group. *P*-values obtained from the fatty acid analysis were adjusted for multiple comparisons by calculation of the false discovery rate (qvalue package). A probability of $P < 0.05$ was considered significant.

3. Results

3.1 Female weight and length

Females from the Low- and High-quality group had similar body weight ($P = 0.149$) and length ($P = 0.315$) at the start of hormonal treatments. There was also no significant correlation between hatching success and female body weight ($R = 0.23$, $P = 0.335$), length ($R = 0.13$, $P = 0.586$).

3.2 Egg floating fraction, fertilization, embryonic survival and hatching success

All females produced floating eggs varying from 1 to 100%. Fertilization of eggs in the floating layer was observed in all 19 batches (Table 1). Fertilization and hatching success ranged from 3 to 99 % and 0 to 80%, respectively. There was no significant correlation between fertilization success and embryonic survival at 30 and 40 hpf or hatching success ($R = 0.48$, $P = 0.060$; $R = 0.48$, $P = 0.059$ and $R = 0.45$, $P = 0.063$, respectively). Fertilization success was similar between Low- and High-quality eggs ($P = 0.129$), whereas embryonic survival at 30 and 40 hpf as well as hatching success (55 hpf) were significantly higher in the High-quality group than in the Low-quality group ($P < 0.001$, Fig. 1).

3.3 Lipid droplet diameter and stage

In the High-quality group ± SD lipid droplet diameter was 80 ± 15 µm, corresponding to a median droplet stage of 5 on the scale of Unuma et al. (2011) at SPE priming, 133 ± 16 µm corresponding to stage 7.5 at DHP, 166 ± 16 µm and stage 9 in unfertilized eggs, and 343 ± 23 µm and stage 10 at 5 hpf. In contrast, oocytes of females in the Low-quality group had a mean ± SD lipid droplet diameter of 119 ± 30 µm and median stage of 6.5 at SPE priming, 178 ± 53 µm and stage 9 at DHP injection, 256 ± 81 µm and stage 10 in unfertilized eggs, and 355 ± 8 µm and stage 10 at 5 hpf. Both lipid droplet diameter and droplet stage were significantly lower in the High-quality group at SPE priming ($P = 0.020$ and $P = 0.025$), at DHP injection ($P = 0.035$ and $P = 0.011$) and in unfertilized eggs ($P = 0.010$ and $P = 0.033$) than in the Low-quality group (Fig. 2). At 5 hpf, lipid droplet diameter was similar in both egg groups ($P > 0.05$) and all samples were at oocyte lipid droplet stage 10.

3.4 Egg dry weight

Eggs in the High-quality group had a mean ± SD dry weigh of 0.063 ± 0.006 mg/egg and eggs from the Low-quality group were 0.060 ± 0.004 mg/egg. This difference was not statistically different (P = 0.356). There was no signifiant correlation between egg dry weight and fertilization (R = 0.28, P = 0.312, embryonic survival at 30 hpf (R = 0.20, P = 0.468), at 40 hpf (R = 0.17, P = 0.538) or hatching success (R = 0.36, P = 0.181).

3.5 Total lipids and fatty acid composition

Egg fatty acid composition is shown in Table 2. There was no significant correlation between total lipid levels in unfertilized eggs and lipid droplet diameter at SPE priming (R = 0.19, $P > 0.05$), DHP (R = - 0.05, $P > 0.05$) or EGG (R = 0.14, $P > 0.05$). In contrast, total lipid levels in unfertilized eggs were negatively correlated with fertilization success (R = - 0.58, P = 0.009) but was neither correlated with embryonic development at 30 hpf (R = - 0.41, $P > 0.05$) and 40 hpf (R = - 0.35, $P > 0.05$) nor with hatching success (R = - 0.45, $P > 0.05$). The average level of total lipids in eggs was not statistically different ($P > 0.05$) between the High-quality (NL = 24.3 ± 5.1 mg and PL = 5.8 ± 1.4 mg) and the Low-quality group (NL = 24.0 ± 6.7 mg and PL = 5.7 ± 0.4 mg).

Egg fatty acid levels in both neutral lipids (NL) and polar lipids (PL) were similar between the two quality groups, including essential fatty acids, ARA, EPA and DHA ($P > 0.05$). There were also no significant correlations between egg fatty acid levels and fertilization success, embryonic survival or hatching success ($P > 0.05$). The most abundant fatty acid in the NL fraction was OA, followed by PA, POA and DHA. In the PL fraction, the most abundant fatty acid was DHA, followed by PA, OA and ARA.

4. Discussion

Reproductive studies involving European eel female eels often apply the SPE primer when oocytes are at maturation stage 4 based on the scale developed by Palstra et al. (2005). Due to limited reproductive success (hatching was not observed), the authors did not relate oocyte characteristics to egg quality. On the other hand, in Japanese eel, specific oocyte stages at induction, based on a lipid droplet-based oocyte stage classification, were associated with resulting egg quality (Unuma et al., 2011). Similarly, results obtained from a recently published study from our group indicated lipid droplet size as a

potential biomarker for development of follicular maturation, and thereby optimization of induction of oocyte maturation in European eel (da Silva et al., 2018). In the present study, a larger sample size revealed that lipid droplet diameter and oocyte stage in the High-quality group were significantly smaller/lower than in the Low-quality group at the time of SPE priming, DHP injection as well as in unfertilized eggs. Overall, this indicates that the outcome of induced follicular maturation and ovulation can be enhanced using the measurement of lipid droplet diameter as criterion for adjusting the timing of hormonal injections. In specific, the range of estimated lipid droplet diameters in oocytes obtained from the High-quality group was 58 to 99 μm (stage 5) at SPE priming and 117 to 160 μm (stage 7.5) at DHP administration. In contrast, diameters of the Low-quality group ranged from 85 to 169 μm (stage 6.5) at SPE priming and 119 to 300 μm (stage 9) at DHP. Therefore, the use of these specific oocyte lipid droplet-based stages (summarized in Fig. 3) may be useful in future efforts timing the induction of follicular maturation. The guidelines, however, differ from those described in Japanese eel, where High-quality eggs (hatching success > 40%) were obtained when follicular maturation was induced at earlier stages of maturation, such as oocyte stage 4 at SPE priming and stage 6 at DHP injection (Unuma et al., 2011). Thus, the results obtained for Japanese eel cannot be directly applied to European eel. The reason why the optimal development of oocytes appears to be at a later stage both at SPE and DHP injection in European eel compared to Japanese eel is unclear. However, possibly due to its longer migration distance, the European eel in general shows slower response to PE injections and requires a longer maturation treatment (15 to >20 weeks compared to 9 to 12 weeks) in Japanese eel (Ohta et al., 1996).

Embryo and yolk-sac larvae viability is also influenced by the nutritional composition of ovulated eggs. High quality eggs must therefore contain all the necessary and optimal nutrient levels for embryo and yolk-sac larvae normal development. Silver eels are then used as model to study egg quality since spawners and early life stages of European eel remain undisclosed in nature. Moreover, silver eels had a natural feeding regime and naturally started the silvering process. Our analysis of egg fatty acid composition showed that, individual fatty acid levels, including essential fatty acids ARA, EPA and DHA, were similar between the High- and Low-quality egg groups. These results support the hypothesis that an optimal nutritional status is required for initiation of female silvering (Larsson et al.,

355 1990). This also indicates that wild-caught female eels are useful models for assessing the levels of
356 essential fatty acids necessary for embryo and yolk-sac larvae normal development.

357 Differences in egg quality between wild and cultured eels (Tomkiewicz et al., 2012) may be influenced
358 by egg fatty acid composition. Our results, showed that ARA levels (2.19 % and 10.03 % in NL and
359 PL, respectively) were considerable higher compared to the ARA levels previously reported in eggs
360 from cultured European eel females (0.58 - 1.13 % and 2.12 - 4.88 %, in NL of PL, respectively)
361 (Støttrup et al., 2016). Similarly, ARA levels in eggs from wild-caught in Japanese eels were also more
362 than six times higher than in eggs from cultured eels (Ozaki et al., 2008). Lower levels of ARA in eggs
363 from cultured fish, in comparison with wild fish, has also been observed in striped bass (Gallagher et
364 al., 1998). Overall, this suggests that egg levels of ARA may be a contributing factor to observed
365 differences in egg quality. In contrast, EPA (1.99 % and 6.64 %, NL and PL, respectively) and DHA
366 levels (6.36 % and 19.49 %, in NL and PL, respectively) were lower than previously reported for eggs
367 obtained from cultured European eel (EPA: 3.06 - 4.71 % and 9.01 - 11.8 %, NL and PL, respectively,
368 and DHA: 9.62 - 11.9 % and 23.5 - 25.4 %, NL and PL, respectively) (Støttrup et al., 2016). Egg levels
369 of EPA and DHA were also lower in eggs from wild-caught compared to cultured Japanese eels (Ozaki
370 et al. 2008). Moreover, the observed DHA/EPA ratio (3.19 in NL and 2.94 in PL) was similar to the
371 previously indicated for eggs from cultured female eels (2.53 to 3.14 in NL and 2.16 to 2.67 in PL)
372 (Støttrup et al., 2016). However, the EPA/ARA ratio (0.91 in NL and 0.66 in PL) was significantly
373 lower than the previously described for eggs obtained from cultured eels (2.71 to 8.01 in NL and 1.85
374 to 5.57 in PL). Thus, a lower EPA/ARA ratio may also contribute to the higher reproductive success
375 generally observed with wild-caught female eels (Tomkiewicz, 2012). Although the composition of
376 eggs from wild-caught females eels may differ somewhat from naturally spawned eggs (effect of
377 hormonal induction), this information can be useful for the formulation of new diets that support the
378 successful reproduction of farmed broodstock.

379 Our results showed no significant differences in egg dry weight between the High- and the Low-quality
380 group. Comparable results were obtained using eggs from cultured female eels (Rozenfeld et al., 2016).
381 Egg dry weight of eggs from wild-caught female eels is also similar to the dry weight estimated in eggs
382 from cultured eels (Rozenfeld et al., 2016). Thus, egg dry weight does not seem a good biomarker for
383 egg quality in European eel. Fertilization success is another conventional egg quality indicator since

eggs with lower quality tend to lose the ability for fertilization quickly (Kjørsvik et al., 1990). In this study, fertilization success was significantly lower in the Low-quality group but not correlated with embryonic survival or hatching success. Thus, it appeared that fertilization success was not a reliable predictor of embryotic survival in European eel as viable larvae were still obtained from batches with relatively low fertilization success. Fertilization success is also a poor indicator of embryonic survival in other fish species, such as turbot (*Scophthalmus maximus*; McEvoy, 1984) and pacific herring (*Clupea harengus pallasi*; Hay, 1986). Still, in Japanese eel, fertilization success seems correlated with hatching success (Furuita et al., 2003).

Conclusions

In this study, the timing of induction of oocyte maturation and ovulation had a determinant effect on the resulting egg quality. This study is the first to describe the relation between oocyte stage at induction and egg quality in European eel. To improve current procedures, we suggest applying oocyte stage classification based on lipid droplet diameter to determine the most favorable timing for the SPE priming and DHP injection. Here, specific guidelines are suggested to enhance timing of the induction of oocyte maturation and ovulation in European eel and minimize the production of low quality eggs due to untimely induction procedures. The analysis of fatty acid composition of eggs obtained from wild-caught silver female eels showed no differences between high and low quality egg groups. Fatty acid levels were, however, considerably different from those previously reported for eggs of cultured European female eels and Japanese eels. Thus, the presented data on egg fatty acid composition provides new insight into the natural levels, which may be used for the development of broodstock diets. Other conventional egg quality markers, such as egg dry weight and fertilization success, seemed poor indicators of egg quality in European eel.

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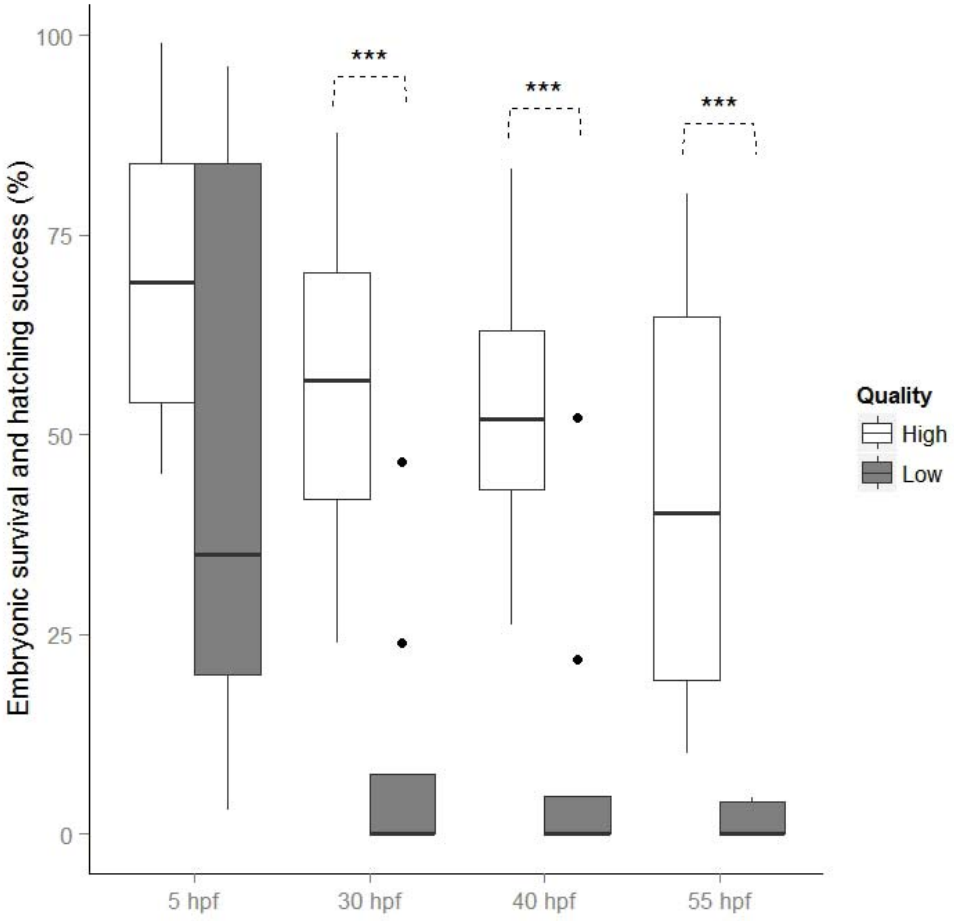
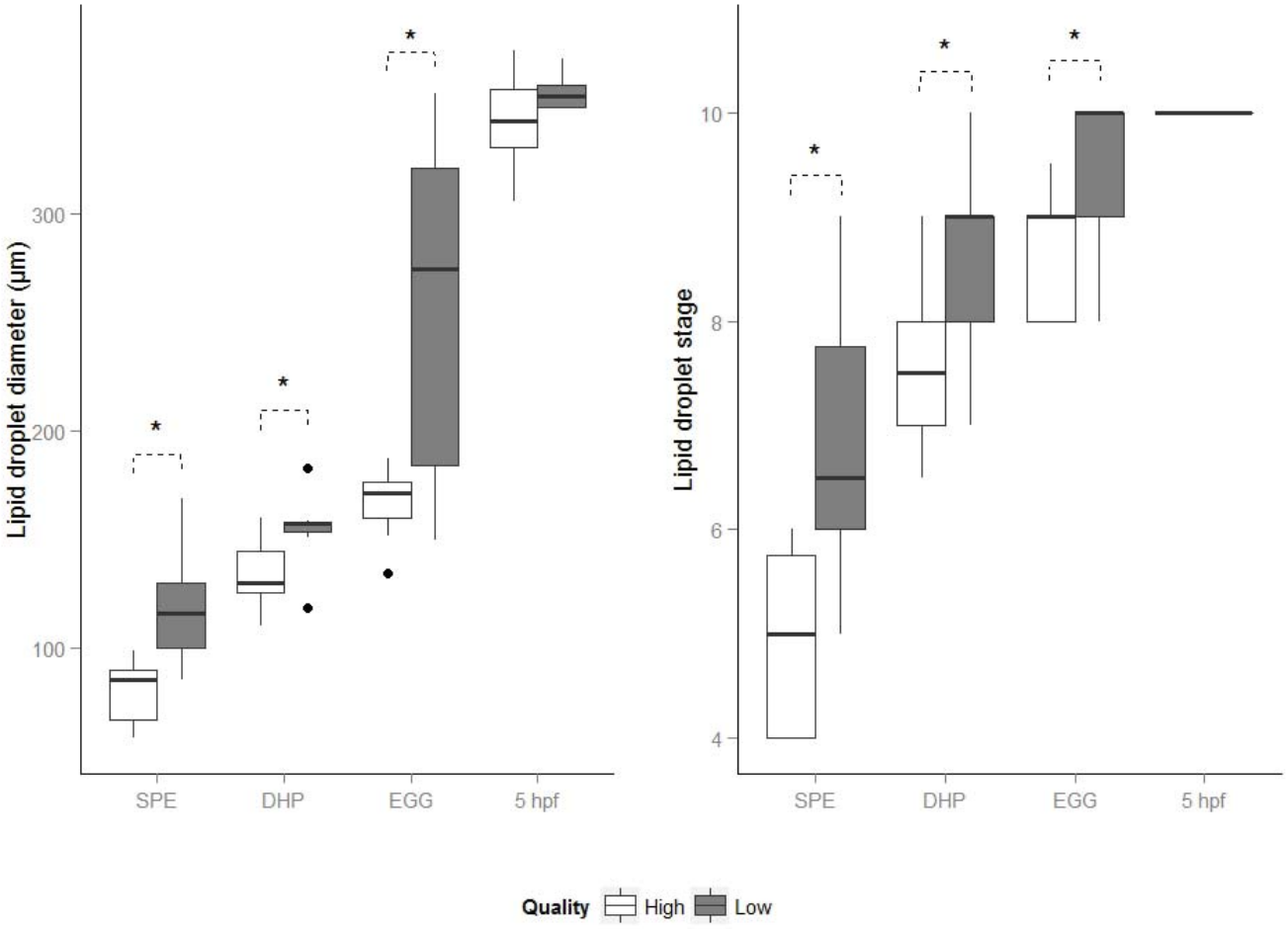
Figure 1. Boxplot showing embryonic survival (%) at ~5 hours post fertilization (hpf, i.e. fertilization success), ~30 hpf, ~40 hpf and ~55 hpf (i.e. hatching success). White and grey boxes indicate values for the High (n = 9) and Low (n = 10) quality groups, respectively. The line inside each box represents the median, the lower and upper sides of each box represent the lower and upper quartile (25 % and 75 %) and whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile distance. Data points outside the boxplot are classed as outliers but were not excluded from the analysis. Significant differences between egg groups are identified with *** for $P < 0.001$.

Figure 2. Boxplot showing lipid droplet diameter (μm) and lipid droplet oocyte stage in samples taken at before SPE priming (SPE), DHP injection (DHP), in unfertilized eggs (EGG) and 5 hours post fertilization (hpf). White and grey boxes indicate embryonic survival and hatching success for the High (n = 9) and Low (n = 10) quality groups, respectively. The line inside each box represents the median, the lower and upper sides of each box represent the lower and upper quartile (25 % and 75 %) and whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile distance. Data points outside the boxplot are classed as outliers but were not excluded from the analysis. Significant differences between egg groups are identified with * for $P < 0.05$.

Figure 3. Pictures of oocytes sampled before SPE priming and DHP injection from female eels delivering high quality eggs. At the bottom, the respective lipid droplet diameter (DD) range, average (μm) and lipid droplet stage (Unuma et al., 2011) is given.


Embryonic survival and hatching success (%)
100
75
50
25
0
***
***
***
5 hpf
30 hpf
40 hpf
55 hpf
Quality
High
Low


Lipid droplet diameter (µm)
100
200
300
SPE
DHP
EGG
5 hpf
*
Lipid droplet stage
4
6
8
10
Quality
High
Low

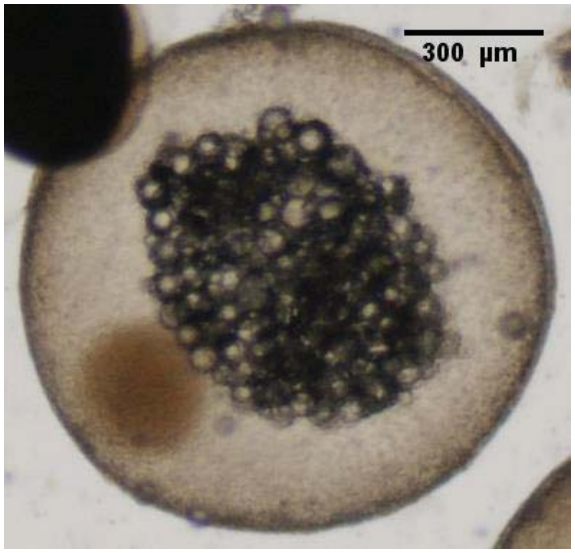
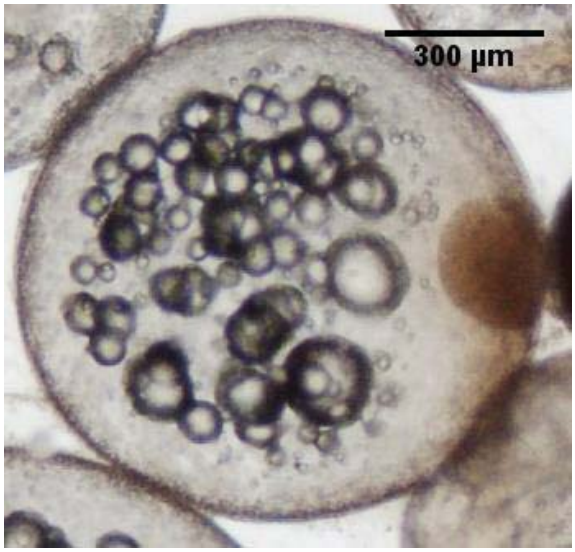
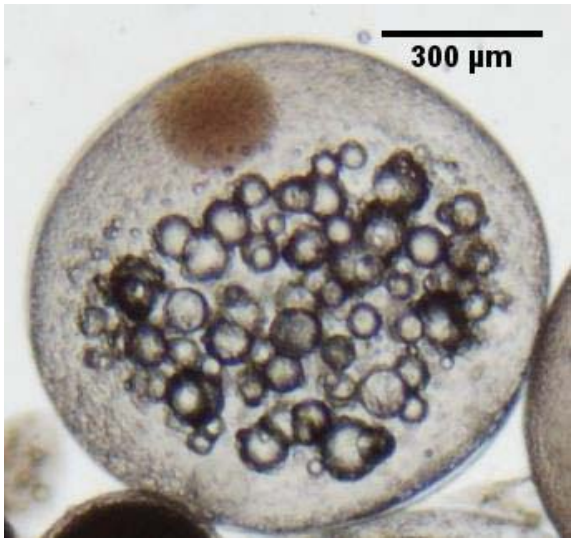
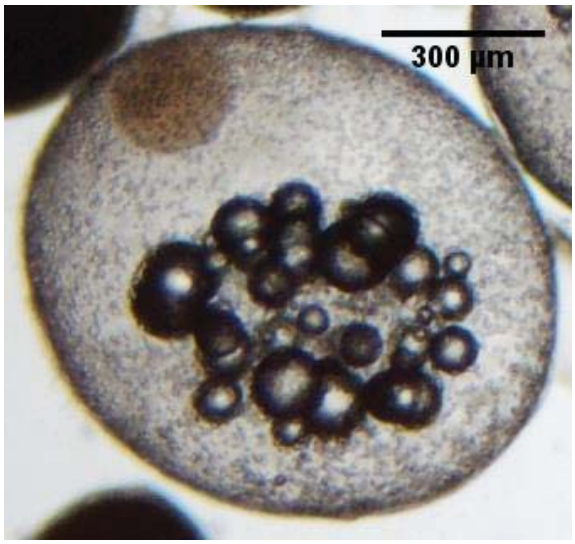
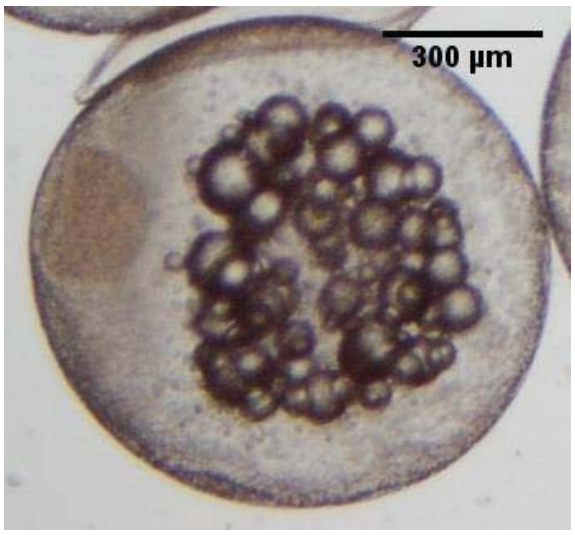
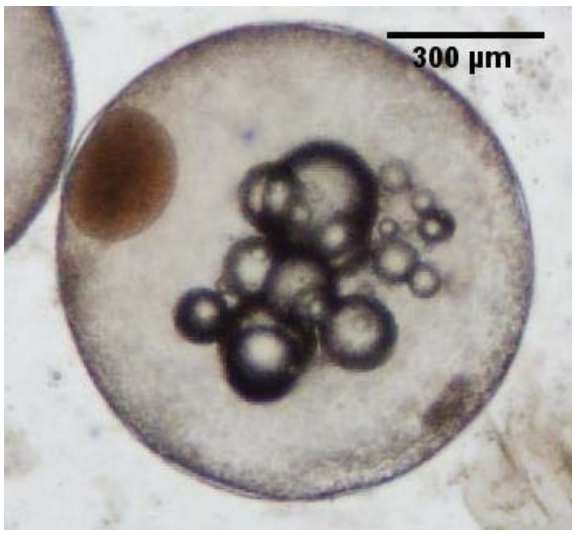
	SPE	DHP
Minimum		
Average		
Maximum		
DD range:	58 - 99 µm	117 - 160 µm
DD mean ± SD:	80 ± 15 µm	136 ± 13 µm
Oocyte stage:	5	7.5

Table 1. Data and quality assignment of egg batches (n=19) from 16 wild-caugth European eel females: Female pittag number, egg batch (first or second stripping), total amount of eggs stripped, floating fraction, fertilization success of the floating fraction, hatching success, larval survival 3 days post hatch (dph). In two cases (D593 first and DD2F), survival was not quantitified and quality assignment was based on longevity.

Female ID	Egg batch no.	Eggs [g]	Floating [%]	Fertilization success [%]	Hatching success [%]	Survival at 3 dph [%]	Longevity (dph)	Egg quality
BA8D	first	324	99	99	80	93	>5	High
D593	first	648	99	54	75	-	>5	High
D593	second	571	50	80	67	78	>5	High
B952	first	322	99	68	65	67	>5	High
D50D	first	229	99	69	55	83	>5	High
C462	first	334	95	45	40	83	>5	High
BA25	first	335	95	97	19	13	>5	High
190A	first	416	85	84	12	63	>5	High
BDF5	first	209	99	54	10	67	>5	High
C670	first	200	90	15	5	0	1	Low
DD2F	first	133	100	96	4	-	1	Low
D389	first	372	25	84	4	0	1	Low
CF19	first	586	99	20	<1	0	0	Low
D262	first	150	88	9	0	0	0	Low
CF34	first	262	1	3	0	0	0	Low
BDF5	second	175	20	88	0	0	0	Low
56DA	first	195	50	25	0	0	0	Low
52BA	second	161	95	81	0	0	0	Low
09A1	first	308	90	35	0	0	0	Low

Table 2. Mean ± SD fatty acid composition (% of total fatty acids) of neutral (NL) and polar lipid (PL) fractions in ovulated eggs from wild-caught silver European female eels.

	Eggs (NL)		Eggs (PL)	
Fatty acid	Avg	SD	Avg	SD
14:0	2.54	0.17	0.88	0.20
14:1	0.34	0.06	0.09	0.10
15:0	0.27	0.02	0.16	0.03
16:0 (PA)	17.58	0.61	18.24	0.83
16:1n-7 (POA)	10.34	1.08	4.40	0.45
16:2n4	0.17	0.12	0.10	0.03
17:0	0.24	0.05	0.35	0.02
16:3n-4	0.62	0.04	0.27	0.03
18:0	3.35	0.28	7.94	0.90
18:1n-9 (OA)	34.73	1.93	15.46	0.88
18:1n-7	6.50	0.55	3.38	0.33
18:2n-6 (LA)	2.13	0.36	1.57	0.56
18:2n-4	0.11	0.03	0.11	0.04
18:3n-6 (GLA)	0.13	0.03	0.14	0.03
18:3n-4	0.95	0.13	0.38	0.06
18:3n-3	0.09	0.01	0.06	0.04
18:4n-3	0.11	0.06	0.11	0.03
20:0	0.41	0.06	0.08	0.01
20:1n-9+n-11	1.32	0.15	0.64	0.18
20:1n-7	0.31	0.04	0.04	0.04
20:2n-6 (EDA)	0.37	0.10	0.34	0.08
20:3n-6	0.33	0.06	0.16	0.33
20:4n-6 (ARA)	2.16	0.16	9.99	0.83
20:3n-3	0.21	0.02	0.12	0.02
20:4n-3	0.36	0.10	0.17	0.04
20:5n-3 (EPA)	2.10	0.36	6.67	1.09
22:5n-3	2.30	0.58	2.26	0.46
22:6n-3(DHA)	6.23	1.01	19.69	1.69
Sum n-3	11.42	1.62	29.48	1.15
Sum n-6	5.06	0.58	12.37	0.98
EPA/ARA	0.94	0.14	0.67	0.11
DHP/EPA	3.30	0.30	3.05	0.65