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Published in:
Aquaculture

Link to article, DOI:
10.1016/j.aquaculture.2020.735581

Publication date:
2020

Document Version
Peer reviewed version

Effects of essential fatty acids and feeding regimes on egg and offspring quality of European eel: Comparing reproductive success of farm-raised and wild-caught broodstock

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Abstract

Production of high-quality offspring from farm-raised broodstock is fundamental to establish a closed-cycle hatchery production of European eel, *Anguilla anguilla*. While development of larval culture technologies progresses, the present study focused on effects of essential fatty acid (EFA) composition of eggs on offspring quality. Three reproduction experiments were conducted, two of which included farm-raised broodstock fed different diets for different periods of time and one wild-caught broodstock, using size-matched females. The formulated diets varied in levels and ratios of three essential fatty acids, arachidonic acid (20:4n-6; ARA), eicosapentaenoic acid (20:5n-3; EPA), and docosahexaenoic acid (22:6n-3; DHA), while feeding periods lasted either 55 or 79 weeks. Dietary influences on egg and offspring fatty acid composition and offspring quality were evaluated and results of the most successful dietary regime was compared to those of wild-caught females. Results showed that elevated dietary levels of ARA were reflected in unfertilized eggs, with high ARA diets significantly increasing the amounts of floating eggs, total lipid content in eggs, fertilization success, and embryonic survival. Further EFA enhancements and prolonged feeding resulted in higher ARA and lower EPA levels in the unfertilized eggs, while DHA levels did not change. Females with prolonged feeding produced offspring of higher quality, i.e. higher egg dry weight and larval survival. Overall, offspring of farm-raised females showed higher EFA levels than those of wild-caught females. However, while fertilization success was comparable, offspring of farm-raised females had significantly lower embryonic survival and hatch success as well as higher proportions of cleavage abnormalities. These results identified embryonic development as the main bottleneck in offspring production from farm-raised females. Once hatched, larval survival and quality was comparable between farm-raised and wild-caught females. Notably,

enhancement of essential fatty acids in female broodstock diets in combination with a long feeding period improved the production of high quality offspring.

Keywords

Anguilla anguilla; broodstock nutrition; assisted reproduction; embryogenesis; cell cleavage

1 Introduction

Aquaculture has experienced remarkable development over the past decades, where it has become the fastest growing food production sector, with ~600 species being cultured worldwide (FAO, 2018). This is largely owed to year-round production and breeding programs enabled by closing the life cycle of targeted species in captivity. In Europe, the impact of aquaculture is increasing, but still it provides only 18% of total seafood consumption, compared to 46% worldwide (FAO, 2018). Here, European aquaculture production has substantial potential to expand through species diversification and domestication, while at the same time reducing pressure on wild populations (COM, 2013; STECF, 2014).

European eel, *Anguilla anguilla*, a high-value species for aquaculture, has lost markets, because it relies on wild-caught glass eels. This, in combination with a general decline in the stock (ICES, 2017; Jacoby and Gollock, 2014), calls for development of breeding and hatchery technology for sustainable aquaculture, as well as conservation measures. However, eels do not reproduce naturally in captivity due to dopaminergic inhibition at the brain-pituitary level impeding sexual maturation (Dufour et al., 2003; Vidal et al., 2004). Nonetheless, gametogenesis and offspring production for the European eel has been realized through hormonal therapy, including extensive research on assisted reproductive

technologies, breeding protocols, and culturing techniques (Mordenti et al., 2014; Palstra et al., 2005; Pedersen, 2004, 2003; Tomkiewicz, 2012; Tomkiewicz et al., 2019). This has led to a stable production of viable offspring entering first-feeding experiments (Butts et al., 2016, 2014; Politis et al., 2018). Even so, variability in egg quality affects viable offspring production, in particular for farm-raised broodstock. For future aquaculture of European eel, hatchery production of high-quality eggs and larvae will be essential for closing the life cycle in captivity.

In fish, egg quality can be defined as the potential for an egg to be fertilized and produce viable offspring (Bobe and Labbé, 2010). Here, high mortality during the embryonic and yolk sac stage tends to impede hatchery production (Kjørsvik et al., 2003; Lazo et al., 2011; Lubzens et al., 2010). Important factors influencing egg quality include (among others) female size (Chambers and Leggett, 1996; Heinimaa and Heinimaa, 2004), age (Berkeley et al., 2004), nutrition (Izquierdo et al., 2001), genetics (Stoddard et al., 2005), origin (Lanes et al., 2012; Salze et al., 2005), environmental conditions (e.g. temperature, photoperiod, salinity) (Aegerter and Jalabert, 2004; Bonnet et al., 2007; Bromage et al., 2001), assisted reproduction techniques (Agulleiro et al., 2006; Mylonas et al., 2010), and stress (Campbell et al., 1992). Of these, nutrition plays a key role, as nutrients required for offspring development are incorporated into the egg prior to or during vitellogenesis (Izquierdo et al., 2001; Migaud et al., 2018). Embryonic demands for fatty acids and amino acids must be met. In particular, long-chain polyunsaturated fatty acids (LC-PUFAs), such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) are essential for structure and composition of cell membranes, organogenesis (i.e. brain, retina, muscle), and/or synthesis of eicosanoid hormones (Glencross, 2009; Sargent et al., 1995; Tocher, 2010). LC-PUFAs are characterized by ≥ 20 carbon atoms and ≥ 3 bonds. Marine teleosts have limited ability to synthesize LC-PUFAs (Sargent et al., 1993) and intake

of essential fatty acids (EFA) occurs mainly through the diet. This includes ARA, EPA, and DHA, important n-3 and n-6 fatty acids, as well as the overall ratio of n-6 to n-3 (Izquierdo et al., 2001; Sargent et al., 1993; Tocher, 2010) as this also may impact early life history traits (Henrotte et al., 2010; Lund and Steenfeldt, 2011; Mazorra et al., 2003; Norberg et al., 2017).

Studies on dietary impacts on eel broodstock reproductive success are limited to Japanese eel female broodstock (Furuita et al., 2007, 2006), European eel female broodstock (Støttrup et al., 2016, 2013), and European eel male broodstock (Baeza et al., 2015a, 2015b; Butts et al., 2019, 2015). The first attempt to develop European eel female broodstock diets was made using the fatty acid composition of wild-caught silver eels as a baseline for enhancement of EFA levels in the diet of farm-raised eels (Støttrup et al., 2013). The study showed that EFA composition in muscle and ovarian tissue could be altered, but that it required a long feeding period leading to gradual changes over 44 weeks (Støttrup et al., 2013). Furthermore, increased ARA content in the broodstock diet elevated ARA levels in the eggs and enhanced the relative frequency of females producing embryos and larvae (Støttrup et al., 2016). Particular to captive reproduction of eels, the integration of dietary components needs to take place prior to induction of sexual maturation and ovarian development. Here, feeding is stopped at the onset of hormonal treatments, mimicking nature where European eels cease feeding concomitant with the onset of silvering and their long spawning migration to reproduce in the Sargasso Sea (Tesch, 2003). Thus, accumulation of lipids in the form of oil droplets in oocytes (lipidation) (Hiramatsu et al., 2015) is initiated during the immature stage, while follicular development is completed, drawing on resources accumulated in muscle, viscera, etc. Therefore, provision of suitable feeds for establishment of high performance farm-raised broodstock must take place during their on-growing period in order to ensure adequate egg quality and offspring viability (Støttrup et al., 2016, 2013).

While striving to close the life cycle of aquaculture species in captivity, egg quality and offspring viability of wild-caught broodstock frequently exceed that of farmed (Hauville et al., 2015; Lanes et al., 2012; Lund et al., 2008; Pickova et al., 1999; Salze et al., 2005). In marine species, studies comparing biochemical composition of eggs from broodstock of different origin have shown distinct differences in EFA (Lanes et al., 2012; Zupa et al., 2017). This also appears to apply to the catadromous eel, where tissue levels of ARA were higher in wild-caught female European eel in the silvering stage than in farm-raised female eels reared on a commercial diet, while farm-raised eels showed higher levels of EPA and DHA than their wild-caught counterparts (Støttrup et al., 2013). Here, wild-caught female eels were used as baseline, considering that natural reproductive and early life history stages remain unknown for this species.

Taking advantage of the progress in reproductive success and offspring production of European eel, this study aimed at i) assessing: the impact of maternal dietary levels of ARA, EPA, and DHA and dietary regimes on reproductive success, egg and offspring quality and ii) comparing EFA, reproductive success and offspring quality of farm-raised females on the best performing diet with wild-caught females, using the latter as benchmark. Here, total lipid and fatty acid composition in eggs and larvae were assessed and egg production, dry weight, fertilization success, embryonic survival, cleavage abnormalities, hatch success, larval survival, and larval morphology were used as offspring quality indicators.

2 Materials and methods

2.1 Ethics statements

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU). Eel experimental protocols were

141 approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food,
142 Agriculture and Fisheries (permit number: 2015-15-0201-00696). Individual fish were
143 anesthetized before tagging, biopsy, and stripping of gametes, and euthanized after stripping
144 (females) or at the end of the experiment (males) using an aqueous solution of ethyl p-
145 aminobenzoate (benzocaine, 20 mg L^{-1}, Sigma Aldrich, Germany). Larvae were anesthetized
146 and euthanized using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany) at a
147 concentration of 7.5 and 15 mg L^{-1}, respectively.

148 2.2 Fish and experimental design

149 2.2.1 Experimental overview

150 Three assisted reproduction experiments were conducted using standardized
151 experimental conditions and selection of size-matched female broodstock. Two reproduction
152 experiments used farm-raised eels fed the three different diets characterized by different EFA
153 levels (Table 1). The first experiment used females fed over a period of 55 weeks (Feeding
154 Trial 1) and the other females fed for 79 weeks with further enhanced diets during the latter
155 period (Feeding Trial 2). The third reproduction experiment included wild-caught female
156 silver eels for comparison among farm-raised and wild-caught females, i.e. broodstock origin.
157 Male broodstock eels in the reproduction experiments were farm-raised eels fed a standard
158 on-growing diet.

159 2.2.2 Diets

160 Broodstock diets were formulated with the purpose to generate three dietary regimes by
161 modifying levels and ratios of ARA, EPA, and DHA in eggs and yolk sac larvae. Diet 1
162 aimed at the highest levels of ARA and DHA and intermediate EPA levels. Diet 2 comprised
163 the lowest ARA level, but the highest EPA and intermediate DHA levels. Therefore, the
164 aimed EPA:ARA ratio was the highest in this diet, while the DHA:EPA ratio was the lowest.

Diet 3 had an intermediate ARA level, while having the lowest EPA and DHA levels. Ingredients and proximate composition are provided in Table 1. The feed was produced as 2 mm extruded pellets at BioMar A/S (Brande, Denmark) in two productions (Table 1). While aiming at similar levels and composition of ARA, EPA, and DHA, capelin ingredients were replaced in the second production due to unavailability of sources. Thus, fish meal NA LT 91.1-91.5 and fish oil NA STD replaced capelin fish meal NA LT (71%) and capelin fish oil NA STD. In order to balance differences in LC-PUFA, DHA Liquid substituted EPAX. From each production, one feed sample per diet was taken at the onset of feeding, and subsequently analyzed for fatty acid composition.

Table 1. Ingredients and proximate composition of Diet 1, Diet 2, and Diet 3 that was fed to European eel, *Anguilla anguilla* broodstock.

Ingredients (%)	**Diet 1_1**	**Diet 1_2**	**Diet 2_1**	**Diet 2_2**	**Diet 3_1**	**Diet 3_2**
Fish Meal NA LT (71%) Capelin	53.4	-	55.5	-	52.5	-
Fish Meal NA LT 91.1-91.5	-	52.8	-	52.2	-	52.3
Fish Peptones, NA Concentrate, CPSP	3.0	3.0	3.0	3.0	3.0	3.0
Wheat, Gluten (min. 80%)	9.0	9.0	6.0	9.0	6.0	9.0
Wheat, Milling quality	17.6	19.0	17.8	18.0	20.6	21.0
Fish Oil, NA STD, Capelin	9.7	-	11.0	-	8.2	-
Fish Oil, NA STD	-	5.3	-	9.2	-	6.8
Rapeseed Oil, Crude	-	2.7	-	2.0	3.2	3.2
Vevodar (35%), DSM	2.1	2.2	1.2	1.3	1.6	1.6
Premix 3053	0.7	0.7	0.7	0.7	0.7	0.7
Mono-calcium Phosphate (MCP)	0.7	1.3	1.6	1.3	1.6	0.3
DL-Methionine (99%)	0.1	0.3	0.1	0.3	0.2	-
Water change	0.2	-1.6	0.1	-1.4	-0.8	-2.8
Lecithin, liquid	0.5	0.5	0.5	0.5	0.5	0.5
EPAX 1050G	2.9	-	2.4	-	2.6	-
DHA Liquid 25/5	-	4.5	-	3.5	-	4.0
Proximate composition (%)						
Moisture	6.5	6.5	6.5	6.5	6.0	5.5
Protein-crude	48.0	48.2	47.0	47.7	47.0	47.8
Fat-crude	23.0	22.1	23.0	23.3	23.0	23.0

Diets labels refer to $_1$: 1st production of feed, $_2$: 2nd production of feed

2.2.3 Feeding trials and broodstock

Female eels for the two feeding trials were reared at Stensgård Eel Farm A/S, Denmark. Stocked as wild-caught glass eels, they were reared in freshwater recirculation aquaculture systems (RAS) on a commercial eel diet (DAN-EX 2848, BioMar A/S) for approximately three years at a temperature of ~23°C. At the onset of the feeding trial, three times ~400 female eels of an average size (length: 63.8 ± 2.4 cm; weight: 520.8 ± 79.7 g) were transferred into three 2800 L tanks, and provided Diet 1_1, 2_1 or 3_1, respectively. The first feeding trial (Trial 1) was completed after 55 weeks, where a proportion of females reached a size range of 60-85 cm length and weight of 600-1200 g (Diet 1_1, n = 26; Diet 2_1, n = 27; Diet 3_1, n = 22). The remaining females in the tanks received prolonged dietary feeding for another 24 weeks, introducing the second production of feeds, Diet 1_2, 2_2, or 3_2. After 79 weeks, the second feeding trial was completed (Trial 2) and females that had reached the same size range were selected (Diet 1_{1+2}, n = 15; Diet 2_{1+2}, n = 18; Diet 3_{1+2}, n = 20).

Wild-caught broodstock included migrating female silver eels (n = 27) caught at Lower Bann, Toomebridge, a donation by the Lough Neagh Fishermen's Co-operative Society, Ireland and selected matching the same size criteria as the farm-raised females. All three reproduction experiments involved farm-raised male broodstock obtained from Stensgård Eel Farm, where they were reared approximately three years on DAN-EX 2848, BioMar A/S at a temperature of ~23°C (1^{st} batch, n = 62, weight = 108.7 ± 12.9 g; 2^{nd} batch, n = 63, weight = 124.9 ± 17.4 g).

2.2.4 Reproduction experiments

For the reproduction experiments, female broodstock were transported in an aerated freshwater tank to the EEL-HATCH experimental facility of the Technical University of Denmark, Hirtshals, Denmark. For the three reproduction experiments, farm-raised females from feeding Trial 1, farm-raised females from feeding Trial 2, and wild-caught females,

were conducted independently following the same assisted reproduction and rearing protocols. Within each experiment, female eels were distributed into two RAS systems each with three 1080 L tanks at a density of 10-15 females per tank; one tank per system was allotted to each dietary treatment per system. Male eels were held in a similar RAS with four tanks (450 L) at a density of ~15 males per tank. Fish were not fed after the transfer. For acclimatization, salinity was gradually increased from 10 to 36 PSU over 14 days using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany). Subsequently, each individual was anaesthetized and tagged with a passive integrated transponder (PIT tag) in the dorsal muscle, and initial length and weight were recorded. At the facility, broodstock were reared at ~20°C and ~36 PSU under 12 h light / 12 h dark photoperiod regime with a 30 min twilight in the morning and evening to resemble the Sargasso Sea. Vitellogenesis was induced in female broodstock by weekly intramuscular injections of salmon pituitary extract (SPE) at 18.75 mg kg^{-1} initial body weight (BW) for 11-21 weeks until weight-increase, indicating initiation of oocyte hydration (da Silva et al., 2018b; Tomkiewicz, 2012). Thereafter, follicular maturation and ovulation was induced, using ovarian biopsies obtained from females under anesthesia to time the injection of 17α,20ß-dihydroxy-4-pregnen-3-one (DHP) at 2 mg kg^{-1} body weight (da Silva et al., 2018b; Ohta et al., 1996; Palstra et al., 2005). Male eels received weekly injections of human chorionic gonadotropin (Sigma-Aldrich, Missouri, USA) at 150 IU/fish (Asturiano et al., 2006; Tomkiewicz, 2012). Prior to spawning, milt from 3-5 males was collected, sperm concentration standardized, and kept in an immobilizing medium (Peñaranda et al., 2010; Sørensen et al., 2013).

Eggs were strip-spawned and fertilized using a standardized sperm to egg ratio (Butts et al., 2014; Sørensen et al., 2016a). After five min, eggs were transferred to 20 L buckets filled with ~15 L of reverse osmosis water salted to ~36 PSU with Red Sea Salt (Red Sea International, Eilat, Israel) at ~19°C. After 60 min, the floating layer of eggs was further

transferred to a second bucket (as above) and kept for 60 min. For each female, the amount of stripped eggs (% of initial weight) was documented. Subsequently, 30 min after fertilization, the amount of floating eggs (%) was determined in a 25 mL volumetric column. Samples of unfertilized eggs (4 × ~100 eggs) were obtained immediately after stripping and frozen at -40°C for lipid and fatty acid analyses. For determination of dry weight of unfertilized eggs (3 x ~200 eggs), samples were kept in an oven at 60°C for 24 h and weighed.

2.2.5 Fertilization success, embryonic development, and hatch success

Eggs were obtained from the floating layer of the separation bucket and incubated in 200 mL sterile tissue culture flasks filled with filtered UV-treated seawater (FUV seawater; filter size: 10, 5, 1 µm) and supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) at 18°C (Politis et al., 2017) and 36 PSU. Here, 3 flasks were stocked with ~2500 eggs to follow embryonic development and an additional 3 flasks were stocked with ~600 eggs to analyze hatch success. For quantification of fertilization success [4 hours post fertilization (hpf)] and embryonic development digital images were taken at 4, 8, 16, 24, 32, 40, and 48 hpf using a Nikon Eclipse 55i microscope equipped with a Nikon digital sight DS-Fi1 Camera. The latter sampling point represents the time shortly before onset of hatching as peak hatch occurs at ~56 hpf at 18°C. Eggs were categorized as fertilized when >4 blastomeres could be observed and fertilization success was calculated as the percentage of fertilized eggs divided by the total number of eggs. Moreover, morphological measurements were conducted at 4 hpf, where total egg area, yolk area, and oil droplet area were measured using NIS Elements image software (Nikon Corporation, Tokyo, Japan). Cleavage abnormalities were determined by counting the number of eggs with regular and irregular cell cleavages. Cleavages were considered abnormal, when cell sizes were uneven or cell adhesion was lacking. Embryonic survival was measured at each sampling point, where the number of dead and alive eggs were counted and expressed as a percentage. Hatch

success was expressed as the number of hatched larvae divided by the total number of stocked eggs.

2.2.6 Larval ontogeny

To monitor larval survival, 20 larvae were kept in triplicate beakers with 90 mL of FUV seawater supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA). Beakers were kept in a temperature incubator at 18°C and 36 PSU (Politis et al., 2017). Every other day, each beaker was checked for mortality and dead larvae were counted and removed. Additionally, larvae were kept in sterile tissue culture flasks filled with FUV seawater and supplemented with the above antibiotic cocktail. At 0 and 5 dph, digital images of 3 × 15 larvae were captured with a Nikon Eclipse 55i microscope equipped with a Nikon Digital Sight DS-Fi1 camera for the following morphological measurements: 1) Standard length (L_S) measured from the lower jaw (excluding protruding teeth) to the tip of the notochord; 2) total body area; and 3) oil droplet area. For determination of dry weight of larvae at 0 and 5 dph (~50 larvae each), samples were kept in the oven at 60°C for 24 h and weighed. Furthermore, 2 × 50 larvae from each female were sampled at 0 and 5 dph and stored at -80°C for fatty acid analyses.

2.3 Lipid extraction and fatty acid composition

Total lipids and lipids for fatty acid composition were extracted from feed samples (~5 g), unfertilized eggs, and larvae at 0 and 5 dph following Folch et al. (1957). In brief, 0.1 mL of unfertilized eggs or ~50 larvae at 0 and 5 dph were diluted in chloroform/methanol (2:1 v/v) and disintegrated with help of sonication in an ice-water bath. Samples were kept at -20°C for 24 h to extract the lipids. Lipids were extracted into pre-weighed vials and evaporated by applying nitrogen. Finally, extracts were weighed on a Mettler Toledo MT5 scale (Mettler Toledo A/S, Glostrup, Denmark; d = 0.1 µg). The amount of total lipid was

calculated as the percentage of dry weight (mg ind^{-1}). For fatty acid composition, a 1 mL mixture of chloroform/methanol (2:1 v/v) was added to the samples with 40 µL internal standard of methyl tricosanoate (C23:0) in chloroform. Samples were placed in an ice-water bath, sonicated in a 2510 Branson ultrasound cleaner for 25-30 min, and subsequently kept for 24 h at -20°C to extract lipids. The sample was then transferred to 1.5 mL autosampler vials with Butyl/PFTE septa screw caps and all liquid evaporated at 60°C by applying a flow of nitrogen from a needle into the mouth of the vial for ~20 min with a 9 port Reacti-Vap Evaporator in a Pierce Reacti-Therm heating module. Thereafter, 1 mL of a toluene, methanol, and acetyl chloride solution (40: 50: 10) was added to the sample and heated for 2 h at 95°C. The vials then received 0.5 mL of aqueous $NaHCO_3$. After shaking the sample, the layer containing the fatty acid methyl esters was removed. The extraction was repeated twice by the addition of 0.5 mL heptane, and the combined sample was added to 2 mL screw top vials with Silicone/PFTE septa and evaporated at 60°C with additional nitrogen flow. Finally, the fatty acid methyl esters were re-suspended in 0.5 mL of chloroform and analyzed by GC-FID consisting of a HP 7890A gas chromatograph (Hewlett-Packard, Palo Aalto, CA, USA) equipped with an Omegawax 320 (30 m 9 3.2 mm 9 0.25 lm) column from Supelco (Bellefonte, PA, USA) using AOCS method Ce 1b-89 (1998). The oven temperature was 15°C min^{-1} to 160°C, hold 2 min, 3°C min^{-1} to 200°C, hold 1 min, and 3°C min^{-1} to 220°C, hold 17 min. A split ratio of 1:50 was used. Fatty acids were subsequently identified by comparison of peaks on a chromatogram with retention times of a mixture of known standards of all fatty acids. Fatty acid content was quantified by calculating the peak area relative to the total area and was expressed as the % fatty acid to the total content of fatty acids.

2.4 Statistical analyses

Data from the three reproduction experiments were analyzed through a series of ANOVA models (Keppel, 1991) using SAS Statistical Software (version 9.4; SAS Institute Inc., Cary, North Carolina). Prior to analysis, residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variances (plot of residuals vs. fitted values). Data deviating from normality or homoscedasticity were $\log_{10}$ or arcsine square-root-transformed. Alpha was set at 0.05. Tukey's analysis was used to compare least-squares means between treatments. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure was fitting the data most appropriately (Littell et al., 1996).

Female ID (individual females and their offspring) was considered random in all models. No significant interactions were detected for any of the tested dependent variables and all models were re-run with the interaction effects removed, analyzing main effects separately (Yossa and Verdegem, 2015). Hence, we analyzed the main effects dietary regime (Diet 1, Diet 2, Diet 3), feeding trial (Trial 1, Trial 2), or broodstock origin (Diet 1 Trial 1, Diet 1 Trial 2, wild-caught) on offspring quality in terms of different dependent variables (Table 2). Model 1 tested the effect of dietary regime (Diet 1, Diet 2, Diet 3) and feeding trial (Trial 1, Trial 2) on parameters for reproductive output and egg quality, while model 2 tested the effect of broodstock origin (best performing diet of Trial 1 and Trial 2, and wild-caught fish; Table 2) on the same dependent variables. If a significant effect was detected for female initial length or weight, a linear regression function was used to test the relationship between length or weight and fertilization success as well as hatch success. Model 3 tested the effects of dietary regime and feeding trial on lipid content and fatty acid composition of unfertilized eggs, while Model 4 tested the effect of broodstock origin on these traits (Table 2).

Furthermore, a series of one-way ANOVA models was used to analyze the fatty acid data in Table A.3 and A.4 for the unfertilized eggs, and larvae at 0 and 5 dph. Model 5 tested

326 the effect of dietary regime and feeding trial on embryonic survival and Model 7 tested
327 parameters characterizing embryonic development. The effect of broodstock origin on the
328 same traits was tested in Model 6 and 8 (Table 2). Moreover, a linear regression function was
329 used to analyze the relationship between cleavage abnormalities at 4 hpf and embryonic
330 survival at 48 hpf. Due to low numbers of hatched larvae, insufficient larval data were
331 obtained for Diet 2 and 3. Therefore, only the effect of broodstock origin on larval survival
332 and morphology was tested in Models 9 and 10, respectively (Table 2).

Table 2. Statistical models and tested effects of dietary regime (Diet 1, Diet 2, Diet 3), feeding trial (Trial 1, Trial 2) and broodstock origin (Diet 1 Trial 1, Diet 1 Trial 2, Wild-caught) on egg quality and offspring performance in European eel, *Anguilla anguilla*

Model	Traits	Dependent variable(s)	Statistical model	Main effect 1 (Levels)	Main effect 2 (Levels)	Main effect 3 (Levels)
1	Reproductive output and egg production	Initial length and weight of females, amount of stripped eggs, amount of floating eggs, dry weight of unfertilized eggs, fertilization success	Series of two-way ANOVAs	Dietary regime (Diet 1, Diet 2, Diet 3)	Feeding trial (Trial 1, Trial 2)	
2	Reproductive output and egg production	Initial length and weight of females, amount of stripped eggs, amount of floating eggs, dry weight of unfertilized eggs, fertilization success	Series of one-way ANOVAs	Origin (Diet 1 Trial 1, Diet 1 Trial 2, Wild-caught)		
3	Lipids and EFA	Total lipid, ARA, EPA, DHA	Series of two-way ANOVAs	Dietary regime (Diet 1, Diet 2, Diet 3)	Feeding trial (Trial 1, Trial 2)	
4	Lipids and EFA	Total lipid, ARA, EPA, DHA	Series of repeated measures mixed-effect model ANOVAs	Origin (Diet 1 Trial 1, Diet 1 Trial 2, Wild-caught)	Age (unfertilized egg, 0, or 5 dph), repeated factor	
5	Embryonic development	Embryonic survival	Three-way repeated measures mixed model ANOVA	Dietary regime (Diet 1, Diet 2, Diet 3)	Feeding trial (Trial 1, Trial 2)	Age (4 to 48 hpf), repeated factor
6	Embryonic development	Embryonic survival	Two-way repeated measures mixed model ANOVA	Origin (Diet 1 Trial 1, Diet 1 Trial 2, Wild-caught)	Age (4 to 48 hpf), repeated factor	
7	Embryonic development	Egg area, yolk area, oil droplet size, cleavage abnormalities at 4 hpf, hatch success	Series of two-way ANOVAs	Dietary regime (Diet 1, Diet 2, Diet 3)	Feeding trial (Trial 1, Trial 2)	
8	Embryonic development	Egg area, yolk area, oil droplet size, cleavage abnormalities at 4 hpf, hatch success	Series of one-way ANOVAs	Origin (Diet 1 Trial 1, Diet 1 Trial 2, Wild-caught)		
9	Larval development	Larval survival	Two-way repeated measures mixed model ANOVA	Origin (Diet 1 Trial 1, Diet 1 Trial 2, Wild-caught)	Age (2 to 14 dph), repeated factor	
10	Larval morphology	Standard length, body area, oil droplet area	Series of two-way repeated measures mixed model ANOVAs	Origin (Diet 1 Trial 1, Diet 1 Trial 2, Wild-caught)	Age (0, 5 dph), repeated factor	

EFA: Essential fatty acids; ARA: arachidonic acid (20:4n-6); EPA: eicosapentaenoic acid (20:5n-3); DHA: docosahexaenoic acid (22:6n-3), hpf: hours post fertilization; dph: days post hatch

337 In total, samples of 46 stripped females were obtained and used in the analyses (Diet 1
338 Trial 1, n = 6; Diet 1 Trial 2, n = 8; Diet 2 Trial 1, n = 3; Diet 2 Trial 2, n = 6; Diet 3 Trial 1,
339 n = 4; Diet 3 Trial 2, n = 6; wild-caught, n = 13). Offspring were monitored throughout
340 ontogeny and survival recorded until 14 days post hatch (dph) or 100% mortality. A detailed
341 description of sample sizes per treatment for each analyses is given in Table A.1.
342

343 # 3 Results

344 ## 3.1 Diets and broodstock

345 Levels of the EFA are summarized in Table 3 for both productions of the diets. In both
346 productions, Diet 1 contained the highest levels of ARA and DHA, while having intermediate
347 EPA levels. Diet 2 had the lowest levels of ARA, the highest EPA and intermediate DHA
348 levels. Diet 3 contained intermediate levels of ARA, while having the lowest EPA and DHA
349 levels. Levels of ARA, EPA (except Diet 1), and DHA in the second production were higher
350 than in the first one. The sum of all monounsaturated fatty acids (MUFAs) was also lower in
351 the second production, while the sum of all PUFAs was higher. Furthermore, the sums of n-3
352 and in particular n-6 fatty acids were higher in the second production of feed among other
353 due to higher levels of 18:2 (n-6). Levels of 18:1 (n-9) were also higher, while 20:1 (n-9, n-
354 11) and 22:1 (n-11) levels were lower in the second feed production. The complete fatty acid
355 composition for both productions of the three diets is shown in Table A.2.
356

357 **Table 3. Essential fatty acid composition (% of total fatty acids) of total lipids extracted**
358 **from production 1 and 2 of the three experimental diets that were fed to European eel,**
359 ***Anguilla anguilla* broodstock.**

	Diet 1_1	Diet 1_2	Diet 2_1	Diet 2_2	Diet 3_1	Diet 3_2
Fatty acid						
ARA	3.91±0.02	4.41±0.01	2.39±0.03	2.72±0.08	3.06±0.00	3.18±0.01
EPA	6.19±0.01	6.11±0.03	6.54±0.09	7.06±0.01	5.60±0.01	6.35±0.03
DHA	9.35±0.02	10.49±0.05	9.08±0.09	10.43±0.03	8.51±0.04	10.13±0.03
EPA:ARA	1.58±0.00	1.38±0.00	2.74±0.00	2.60±0.07	1.83±0.00	2.00±0.01

DHA:EPA	1.51±0.01	1.72±0.00	1.39±0.01	1.48±0.00	1.52±0.00	1.60±0.00
SUM MUFA	46.28±0.46	32.72±0.02	49.05±0.43	33.92±0.17	48.75±0.16	32.86±0.14
SUM PUFA	30.34±0.18	40.19±0.01	27.93±0.12	37.39±0.04	30.64±0.12	39.77±0.16
SUM n-3	19.16±0.01	22.31±0.01	19.29±0.12	23.62±0.01	17.22±0.04	22.51±0.11
SUM n-6	9.15±0.15	16.84±0.00	6.77±0.04	12.65±0.03	10.33±0.12	16.20±0.01
n-6:n-3	0.48±0.01	0.75±0.00	0.35±0.00	0.54±0.00	0.60±0.01	0.72±0.00

Values represent average ± SD; ARA: arachidonic acid (20:4n-6); EPA: eicosapentaenoic acid (20:5n-3); DHA: docosahexaenoic acid (22:6n-3), MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; $_1$: 1st production of feed, $_2$: 2nd production of feed

3.2 Female broodstock traits and egg production

Initial length of stripped females did not differ between diets ($p = 0.152$) nor between Trial 1 and Trial 2 across the diets ($p = 0.214$; Table 4; Model 1). Overall, initial body weight of the selected females of the three different dietary regimes was also similar ($p = 0.089$), while females from Trial 1 weighed on average more than those from Trial 2 ($p = 0.013$). However, neither fertilization success ($R^2 = 0.004$, $p = 0.781$) nor hatch success ($R^2 = 0.05$, $p = 0.275$) were related to initial female weight. Thus, female body weight was not included as a potential factor influencing offspring quality in this study.

374 **Table 4. Characteristics of European eel, *Anguilla anguilla* broodstock, eggs, and offspring from females fed on three different diets**
375 **during Trial 1 and Trial 2, and Wild-caught females.**

	Diet 1 Trial 1	Diet 1 Trial 2	Diet 2 Trial 1	Diet 2 Trial 2	Diet 3 Trial 1	Diet 3 Trial 2	Wild-caught
Initial length – all females (cm)	73.6±3.8	72.1±4.0	73.9±3.6	75.1±5.1	74.0±4.3	73.1±4.7	76.2±1.3
Initial weight – all females (g)	944±145	816±137	905±126	887±177	934±148	847±139	950.6±41
Initial length – stripped females (cm)	71.4±1.9	70.6±1.2	74.0±2.5	75.0±2.3	77.8±1.5	71.2±2.4	74.2±1.4
Initial weight – stripped females (g)	869±48	790±26	919±47	895±59	1071±42	827±64	951±52
Stripped eggs (% initial weight)	37.7±4.8	33.24±4.7	36.3±5.1	30.5±4.3	33.7±1.6	29.1±2.6	38.1±4.3
Floating eggs (%)	88.3±8.8	70.6±13.2	35.7±19.5	67.3±17.1	52.5±18.3	32.8±13.9	74.1±11.4
Fertilization success (%)	69.35±15.78	61.49±12.49	37.46±0.60	46.55±13.34	23.28±9.28	57.58±30.49	57.02±23.11
Hatch success (%)	3.02±2.78	7.81±11.01	0.00±0.00	2.42±2.22	4.17±6.65	0.47±0.66	40.57±34.09
Dry weight (mg egg^{-1})	0.053±0.003	0.062±0.002	0.056±0.000	0.062±0.002	0.059±0.001	0.062±0.003	0.059±0.003
Dry weight (mg 0 dph $larva^{-1}$)	0.058±0.004	0.062±0.001	n.d.	n.d.	n.d.	n.d.	0.063±0.001
Dry weight (mg 5 dph $larva^{-1}$)	0.055±0.002	0.063±0.002	n.d.	n.d.	n.d.	n.d.	0.063±0.001

376 Values represent average ± SD; n.d. no data available

The amount of stripped eggs (% initial weight) did neither differ between females from the different dietary regimes ($p = 0.586$) nor between females from Trial 1 and Trial 2 ($p = 0.161$; Table 4; Model 1). In contrast, the amount of floating eggs was higher for females fed Diet 1 compared to those fed Diet 3 ($p = 0.049$) and intermediate for females reared on Diet 2, with no difference between feeding trials ($p = 0.672$). Dry weight of unfertilized eggs was not related to dietary regime ($p = 0.582$), while eggs of females from Trial 2 had higher dry weight than those of Trial 1 ($p = 0.006$; Table 4).

Initial weight of females of different broodstock origin did not differ ($p = 0.057$), while initial length differed with wild-caught eels being larger than Diet 1 Trial 2 ($p = 0.024$), whereas Diet 1 Trial 1 females showed intermediate values (Table 4, Model 2). There was no relationship between initial length and fertilization success ($R^2 = 0.02$, $p = 0.621$), or hatch success ($R^2 = 0.02$, $p = 0.601$). Thus, female length was not included as a potential factor influencing offspring quality. Likewise, the amount of stripped eggs ($p = 0.696$) and the amount of floating eggs ($p = 0.593$) did not differ among Diet 1 Trial 1, Diet 1 Trial 2, and wild-caught females (Table 4). On the contrary, dry weight of unfertilized eggs as well as larvae at 0 and 5 dph depended on broodstock origin, with the lowest values for offspring of Diet 1 Trial 1, while higher values were found for offspring of Diet 1 Trial 2 and those of wild-caught origin ($p = 0.008$). Within trials, dry weight did not change over time from unfertilized eggs to larvae ($p = 0.377$; Table 4).

3.3 Total lipids of eggs and larvae

Total lipid content (% dry weight; Fig. 1) of unfertilized eggs differed among dietary regimes ($p = 0.033$), such that eggs from females reared on Diet 1 had significantly higher lipid content than those from females reared on Diet 3, whereas Diet 2 eggs were

401 intermediate (Fig. 1A; Model 3). On the other hand, total lipid content was similar for
402 females from Trial 1 and Trial 2 ($p = 0.486$; Fig. 1B). Moreover, total lipid in unfertilized
403 eggs, larvae at 0 dph, and larvae at 5 dph did not differ among Diet 1 Trial 1, Diet 1 Trial 2,
404 and wild-caught females (Fig. 1C; Model 4). Since no significant interaction was observed,
405 the main effects for the groups were combined and are shown in the figure 1D, where larvae
406 at 5 dph showed significantly lower lipid content than unfertilized eggs and newly hatched
407 larvae ($p < 0.0001$; Fig. 1D).
408

409 ## 3.4 Fatty acid composition in eggs and larvae

410 Unfertilized eggs reflected dietary regime ($p < 0.0001$), where eggs from females
411 reared on Diet 1 had the highest relative ARA levels and those of Diet 2 the lowest (Fig. 2A;
412 Model 3). Notably, eggs obtained from females of Trial 2 had higher ARA levels than those
413 of Trial 1 ($p = 0.007$; Fig. 2B). Similarly, dietary regime ($p = 0.012$) affected EPA levels of
414 unfertilized eggs, whereas eggs from females reared on Diet 2 showed higher EPA levels
415 than eggs from those reared on Diet 3, while values for eggs obtained from females fed Diet 1
416 were intermediate (Fig. 2C). In contrast, EPA levels of eggs from Trial 2 females were lower
417 than those from Trial 1 females ($p = 0.040$; Fig. 2D). Moreover, DHA levels of unfertilized
418 eggs differed between the dietary regimes ($p = 0.006$), such that those from females reared on
419 Diet 2 had higher DHA content than those obtained from Diet 1 or Diet 3 (Fig. 2E), while
420 eggs from Trial 1 and 2 females did not differ in this respect ($p = 0.163$; Fig. 2F). The relative
421 fatty acid content of unfertilized eggs from the seven groups of females is given in Table A.3.
422 The relative ARA levels of unfertilized eggs, larvae at 0 dph, and larvae at 5 dph also
423 differed among Diet 1 Trial 1, Diet 1 Trial 2, and wild-caught broodstock. Since no
424 significant interaction was observed, the main effects for the groups were combined. Thus,
425 the highest ARA levels related to eggs from Trial 2 females reared on Diet 1, and the lowest

426 to wild-caught female eggs ($p < 0.0001$; Fig. 2G; Model 4), while ARA levels were similar in
427 unfertilized eggs and larvae at 0 dph, but relatively higher in larvae at 5 dph ($p < 0.0001$; Fig.
428 2H). Similarly, main effects for the groups were combined for EPA levels and were higher in
429 eggs and offspring of females reared on Diet 1 compared to those of wild-caught ($p < 0.0001$;
430 Fig. 2I), while no difference was found between unfertilized eggs, larvae at 0 dph, and larvae
431 at 5 dph ($p = 0.287$; Fig. 2J). Furthermore, no significant interaction was observed for DHA
432 levels and the main effects for the groups were combined. Thus, eggs and larvae of farm-
433 raised females in Trial 1 fed Diet 1 showed highest DHA levels, while wild-caught females
434 showed lowest ($p < 0.0001$; Fig. 2K). DHA levels were similar for unfertilized eggs and
435 larvae at 0 dph (Diet 1 Trial 1, Diet 1 Trial 2, wild-caught broodstock), while the relative
436 content was higher in larvae at 5 dph ($p < 0.0001$; Fig. 2L). The relative fatty acid
437 composition of larvae at 0 and 5 dph from the three groups of females is given in Table A.4.
438 Overall, eggs and larvae from farm-raised females fed Diet 1 showed higher amounts of
439 PUFA, while certain saturated fatty acids and MUFA levels were lower than in those of wild-
440 caught females (Tables A.3 and A.4). For instance, the levels of palmitoleic acid, 16:1 (n-7),
441 oleic acid, 18-1 (n-9), and cis-vaccenic acid, 18-1 (n-7) were consistently lower in eggs as
442 well as larvae at 0 and 5 dph in offspring from farm-raised females fed Diet 1 compared to
443 those of wild-caught females. During the first 5 dph, saturated fatty acid and MUFA levels of
444 Diet 1 Trial 1 and 2, as well as wild-caught females decreased slightly, while PUFA levels
445 increased in the percentage of total fatty acids. The sum of n-3 and n-6 fatty acids were
446 higher in eggs and larvae obtained from farm-raised females fed Diet 1, showing a higher n-6
447 to n-3 ratio compared to those of wild-caught. Comparing eggs of farm-raised females, eggs
448 obtained from females fed Diet 2 had the lowest n-6 to n-3 ratio. The EPA:ARA ratio was
449 lowest in Diet 1, in particular Diet 1 Trial 2, and highest in Diet 2. Throughout life stages, i.e.

450 unfertilized eggs, larvae at 0 and 5 dph, the EPA:ARA was similar between wild-caught and
451 Diet 1 offspring.
452

453 3.5 Fertilization success

454 Dietary regime affected fertilization success (Model 1). In this case, fertilization
455 success of eggs related to Diet 1 females was higher than for Diet 2 and 3 ($p = 0.011$; Fig
456 3A), while there was no difference between Trial 1 and Trial 2 ($p = 0.543$; Fig. 3B).
457 Moreover, the fertilization success of eggs from females fed Diet 1 in Trial 1 and 2 was
458 similar to wild-caught broodstock ($p = 0.483$; Fig. 3C; Model 2).
459

460 3.6 Embryonic development

461 3.6.1 Survival

462 Similar to fertilization success, embryonic survival differed between the three dietary
463 regimes, such that embryos obtained from females reared on Diet 1 showed the highest
464 survival ($p < 0.0001$; Fig. 4A; Model 5), while no effect of feeding trial on embryonic
465 survival was detected ($p = 0.902$; Fig. 4B). During embryonic development, the survival rate
466 declined slightly from 4 to 8 hpf, followed by a steep decline from 8 to 16 hpf after which
467 survival stabilized ($p < 0.0001$; Fig. 4C). Furthermore, embryonic survival varied
468 considerably among offspring from individual females in particular for Diet 3 (Fig. 4D-F).
469 Furthermore, embryonic survival was lower for Diet 1 females than for wild-caught
470 females ($p < 0.001$; Fig. 4G; Model 6). As above, embryonic mortality was highest in the
471 early stages and stabilized thereafter ($p < 0.0001$; Fig. 4H). The variability among offspring
472 from individual females was high for embryonic survival, especially for the wild-caught
473 broodstock (Fig. 4I-K). Notably, offspring from farm-raised broodstock fed Diet 1 showed

474 the previously observed decline in survival between 8 and 16 hpf. In contrast, wild-caught
475 broodstock with >50% fertilization success at 4 hpf had consistently higher survival
476 throughout development (Fig. 4K).
477

478 **3.6.2 Morphology**

479 Morphological characteristics of embryos at 4 hpf did not differ among offspring
480 derived from different dietary regimes in terms of egg area (Diet 1: 1.46 ± 0.08 mm^2, Diet 2:
481 1.54 ± 0.11 mm^2, Diet 3: 1.09 ± 0.16 mm^2; p = 0.08), yolk area (Diet 1: 0.67 ± 0.01 mm^2,
482 Diet 2: 0.66 ± 0.01 mm^2, Diet 3: 0.65 ± 0.02 mm^2; p = 0.814), and oil droplet area (Diet 1:
483 0.098 ± 0.001 mm^2, Diet 2: 0.1 ± 0.002 mm^2, Diet 3: 0.105 ± 0.003 mm^2; p = 0.139; Model
484 7). Neither did these measures differ between feeding trials, i.e. egg area (Trial 1: 1.32 ± 0.10
485 mm^2, Trial 2: 1.41 ± 0.08 mm^2; p = 0.479), yolk area (Trial 1: 0.65 ± 0.01 mm^2, Trial 2: 0.67
486 ± 0.01 mm^2; p = 0.333), and oil droplet area (Trial 1: 0.099 ± 0.002 mm^2, Trial 2: 0.103 ±
487 0.002 mm^2; p = 0.168). Data at 48 hpf were excluded from these analyses as the number of
488 embryos available was insufficient.

489 Embryonic morphology at 4 hpf also did not differ among broodstock origin in terms of
490 egg area (Diet 1 Trial 1: 1.40 ± 0.14 mm^2, Diet 1 Trial 2: 1.53 ± 0.14 mm^2, wild-caught: 1.74
491 ± 0.14 mm^2, p = 0.271), yolk area (Diet 1 Trial 1: 0.65 ± 0.02 mm^2, Diet 1 Trial 2: 0.68 ±
492 0.02 mm^2, wild-caught: 0.65 ± 0.02 mm^2, p = 0.405), and oil droplet area (Diet 1 Trial 1:
493 0.096 ± 0.002 mm^2, Diet 1 Trial 2: 0.099 ± 0.002 mm^2, wild-caught: 0.101 ± 0.002 mm^2, p =
494 0.144; Model 8). Also, egg area at 48 hpf was similar (Diet 1 Trial 1: 1.53 ± 0.16 mm^2, Diet 1
495 Trial 2: 1.69 ± 0.16 mm^2, wild-caught: 1.81 ± 0.16, p = 0.446).

3.6.3 Cleavage abnormalities

Abnormal cleavage patterns of embryos, recorded at 4 hpf, included uneven cell sizes or lack of adhesion among cells, resulting in cells principally moving freely (Fig. 5A). These cleavage abnormalities occurred on average less frequently in embryos from females fed Diet 1 than Diet 2 and 3, however, female variability was high and no significant effects of dietary regime ($p = 0.059$; Fig. 5B) nor feeding trial was found ($p = 0.121$; Fig. 5C; Model 7). Moreover, the proportion of embryos with cleavage abnormalities was higher for Diet 1 Trial 1 females than for wild-caught broodstock ($p = 0.013$, Fig. 5D; Model 8), while Diet 1 Trial 2 did not differ significantly. A highly significant relationship between abnormalities and survival at 48 hpf was found when pooling data from all females independent of origin (Fig. 5E). No significant relationship was found for embryos from the farm-raised females fed Diet 1 in Trial 1 individually (Fig. 5F), while cleavage abnormalities were related to embryonic survival for farm-raised females fed Diet 1 in Trial 2 (Fig. 5G) and wild-caught females (Fig. 5H).

3.7 Hatch success

Hatch success was neither related to broodstock dietary regime ($p = 0.409$; Fig. 6A) nor to feeding trial ($p = 0.432$; Fig. 6B; Model 7). However, hatch success obtained from wild-caught females were 8-fold higher than for Diet 1 Trial 2 and 13-fold higher than for Diet 1 Trial 1 females fed Diet 1 ($p = 0.014$; Fig. 6C; Model 8).

3.8 Larval development

Numbers of hatched larvae for Diet 2 and 3 were limited, therefore statistical analyses of larval development were only conducted for larvae obtained from Diet 1 Trial 1 and 2 and

wild-caught females. Early larval development of European eel during the yolk sac phase ranges from newly hatched larvae to larvae that commence exogenous feeding around 12-14 dph (Fig. 7A). Larval survival was higher for larvae from Diet 1 Trial 2 females compared to Trial 1, while larval survival from wild-caught females was intermediate ($p < 0.0001$; Fig. 7B; Model 9). Generally, larval survival decreased over time with the highest survival at 2 and 4 dph and the lowest at 14 dph ($p < 0.0001$; Fig. 7C). However, variability was high amongst individual female offspring depending on origin (Fig. 7D-F). Although limited in numbers, larvae from Diet 1 Trial 2 females showed the most stable survival throughout development (Fig. 7E) with levels corresponding to the upper range of the wild-caught (Fig. 7F). In contrast, larvae from Diet 1 Trial 1 females showed a drastic decline in survival from 4 to 8 dph and hardly any survival at 14 dph (Fig. 7D). Larval standard length also depended on broodstock origin ($p = 0.011$; Fig. 7G; Model 10), where larvae obtained from Diet 1 Trial 2 females were longer than those from Trial 1, while larvae from wild-caught females were intermediate. In general, larval standard length doubled over time from hatch to 5 dph ($p < 0.0001$; Fig. 7H). Likewise, body area related to broodstock origin, with the biggest larvae obtained from Diet 1 Trial 2 females, which were larger than those of wild-caught females ($p = 0.037$; Fig. 7I), while those of Diet 1 Trial 1 females were intermediate. Overall, body area more than doubled from hatch to 5 dph ($p < 0.0001$; Fig. 7J). In contrast, oil droplet size decreased during the yolk sac stage in all treatments ($p < 0.0001$; Fig. 7L) with no impact of broodstock origin ($p = 0.262$; Fig. 7K).

4 Discussion

543 In this study, we tested the effects of enhanced broodstock diets and two feeding
544 periods on egg characteristics and early offspring performance from farm-raised European eel
545 broodstock. The results of the best performing diet (Diet 1) were then compared to wild-
546 caught broodstock, as benchmark. Overall, using size-matched broodstock in assisted
547 reproduction experiments, this is the most comprehensive study to quantify maternal
548 nutritional effects on egg composition and offspring performance of anguillid eels.
549 Specifically, we report several key findings: (1) Diets enhanced in EFA composition
550 increased the total lipid content of eggs, the amount of floating eggs, fertilization success, and
551 embryonic survival; (2) longer feeding duration and further EFA enhancement led to higher
552 egg ARA levels and dry weight as well as improved larval survival; (3) low survival during
553 the maternal-to-zygotic transition phase (8 to 16 hpf) impeded larval production, especially
554 for the farm-raised broodstock; and (4) larvae from broodstock fed EFA enriched diets with
555 prolonged feeding reached similar quality as those of their wild-caught counterparts.

556 Among the farm-raised females, the manipulation of EFA in the diet influenced egg
557 total lipid, the proportion of floating eggs, fertilization success and embryonic performance.
558 Thus, the total lipid content of eggs from females fed Diet 1 was higher than those of Diet 2
559 and 3 independent of production and feeding duration and despite similar lipid levels in the
560 diets. In Japanese eel, high quality eggs from females fed a commercial diet were correlated
561 to low total lipid levels in unfertilized eggs (Furuita et al., 2006, 2003). However, in the
562 present study, total lipid levels in Diet 1 did not exceed the levels of high quality eggs in the
563 aforementioned Japanese study, indicating that the levels reached in Diet 1 approached the
564 optimum. In accordance, the obtained lipid levels in eggs from farm-raised females on the
565 best performing diet were similar to those of wild-caught females in our study.

566 Moreover, the fatty acid composition of the egg lipid resource affects offspring
567 performance emphasizing the importance of EFA in broodstock nutrition (Sargent et al.,

568 1995). In the current study, increased levels of ARA in Diet 1 increased egg ARA content
569 and improved fertilization success and embryonic survival, which compares to results for
570 other species with marine larvae. For instance, in Atlantic halibut, *Hippoglossus*
571 *hippoglossus*, (Mazorra et al., 2003) and Atlantic cod (Røjbek et al., 2014) broodstock fed
572 ARA enhanced diets produced offspring with higher fertilization and hatch success than
573 broodstock fed lower ARA levels. Similarly, for European sea bass, *Dicentrarchus labrax*,
574 embryos obtained from females fed an ARA enriched diet had significantly higher embryonic
575 survival at 48 hpf (Bruce et al., 1999). Hereby, the study extends, previous results on
576 European eel showing that i) ARA levels in the muscle and ovary can be enhanced through
577 enhanced dietary EFA composition in the diet prior to induced gonadal development
578 (Støttrup et al., 2013); and ii) that feeding high dietary ARA levels for 24 weeks prior to
579 induction of development increased the prevalence of females/stripped egg batches resulting
580 in fertilized eggs, embryo and larvae, measured on a binomial scale (Støttrup et al., 2016).
581 However, too high levels may hamper egg quality. In Japanese eel, ARA levels between 2.8
582 and 4.0 % of total FA in the unfertilized eggs represented high quality, whereas too high
583 ARA levels (4.6 – 5.6 % of total FA) could be detrimental to offspring performance (Furuita
584 et al., 2007, 2006). In this context, dietary ARA at the highest levels (3.32 % of total FA in
585 unfertilized eggs) in the present study represented the high quality category found in Japanese
586 eel and relative ARA contents at this level similarly appeared to promote offspring
587 developmental competence and larval survival in European eel. In particular, the ARA level
588 attained in the eggs, embryos and larvae from Diet 1 females exceeded that of wild-caught
589 females in contrast to the previous study of Støttrup et al. (2013).

590 Levels of EPA and DHA in broodstock diets may also affect egg quality and offspring
591 performance. For instance, in gilthead seabream, *Sparus aurata* EPA levels were positively
592 correlated with egg fertilization rates (Fernandez-Palacios, 1995), while in other cases too

high levels may hamper reproductive success, as EPA may compete with ARA (Sargent et al., 1999a). Thus, in anguillid species, decreasing EPA levels by intake of dietary lipids have been found to enhance egg quality (Furuita et al., 2007; Støttrup et al., 2016) indicating EPA might have been supplied in excess. In the present study, intermediate EPA levels in the best performing diet, Diet 1, were reflected in the unfertilized eggs. Still, levels may benefit from some adjustment as the EPA levels were higher than in the unfertilized eggs obtained from wild-caught females. On the other hand, DHA levels have been positively correlated to egg quality parameters in Japanese eel (Furuita et al., 2006). In the current study, DHA levels also were highest in Diet 1, however in the unfertilized eggs, highest levels were found in eggs obtained from females fed Diet 2. Nonetheless, the better performance of offspring from Diet 1 indicated that DHA levels in this diet were sufficient or might not affect egg quality as crucially as ARA in this case, not least taking into account that DHA levels in offspring from Diet 1 females were still higher than those from wild-caught females.

In addition to the EFA levels, their ratios are crucially important in broodstock nutrition (Bell et al., 1997). Here, ratios of DHA/EPA are recommended to be >1 and EPA/ARA <3, which was the case in all diets used in this study. Moreover, the overall n-3 to n-6 ratio might be of importance, which has been shown in Japanese eel, where a too high ratio of n-6 to n-3 fatty acids had a negative impact on embryonic development (Furuita et al., 2007). However, the ratio in the diets leading to lower offspring quality in Japanese eel was considerably higher (n6:n3: 2.2) than in our study (highest n6:n3 ratio 0.75) and feeding duration may similarly affect results.

The females in the present study, which required an extended feeding period to reach the same size, while receiving further enhanced diets, accumulated more ARA and produced egg and offspring of higher quality, considering unfertilized eggs up to the larval stage. Due to the increase in EFA in the second feed production, it cannot be concluded from this

618 experiment, if the females accumulated ARA in a more efficient way or the increase in the
619 ARA content of the eggs was a direct dietary effect. In support of the first interpretation, two
620 previous studies found a selective accumulation of ARA over time and importance of long
621 feeding periods in European eel (Støttrup et al. 2013; 2016). At the same time, the differences
622 in EFA, including higher ARA levels, in the second production may have contributed to the
623 higher quality of offspring from Trial 2 females. In light of this, the size-matched approach
624 applied in this study added new insights into the interaction between dietary effects and
625 feeding duration of interest in broodstock management, while future experiments are needed
626 to disentangle effects of diets, growth, and feeding duration. In this context, it is worth
627 considering that eels in nature build up resources and cease feeding prior to spawning
628 migration and presumably their reproduction.

629 In diadromous, semelparous fishes, reproductive strategies may be a trade-off between
630 growth and reproduction, which in eels may lead to differences in age and size at the onset of
631 spawning migration (Yokouchi et al., 2018). It is still questioned as to whether eels reach the
632 migration stage at the earliest possible point as suggested by Svedang et al. (1996) or may
633 risk spending extra time in their growth habitats under good conditions to reach a larger body
634 size (Yokouchi et al., 2018). Certainly, eels show extensively varying growth rates and
635 flexible timing of silvering (Bevacqua et al., 2012; Vøllestad, 1992; Yokouchi et al., 2018).
636 This also applies to aquaculture under controlled conditions, where growth rates differ
637 substantially. While fast growth is commonly targeted in aquaculture, it may not necessarily
638 favor broodstock performance due to trade-offs in allocation of resources to growth and
639 reproduction (Folkvord et al., 2014), as also indicated in the present study.

640 The size-matched females fed enhanced diets over the prolonged feeding period
641 furthermore produced eggs of higher dry weight. Egg size and dry weight are often
642 influenced by maternal size and used as quality indicators, as they affect early life history in

643 marine fishes (Bobe and Labbé, 2010; Kjørsvik et al., 1990; Rideout et al., 2005; Trippel,
644 1998). For instance, in Atlantic cod, egg dry weight and fecundity was lower in poor
645 condition females (Lambert and Dutil, 2000; Ouellet et al., 2001). On the other hand, egg dry
646 weight was negatively correlated to cell clarity (Penney et al., 2006). Previous studies on
647 European eel did not find any relation between dry weight and offspring quality (da Silva et
648 al., 2018a; Rozenfeld et al., 2016). However, the results of this more comprehensive study
649 indicate that dry weight might play a role in defining embryonic developmental competence
650 and thus, dry weight in combination with EFA levels may prove useful as quality indicators
651 in European eel.

652 In accordance with these egg quality parameters, the larvae obtained from farm-raised
653 broodstock fed Diet 1 with prolonged feeding reached similar quality as those of their wild-
654 caught counterparts. Notably, once hatched, larval survival was comparable between wild-
655 caught and farm-raised females fed Diet 1 for the prolonged feeding period, and the body
656 area of larvae from these farm-raised females was even significantly higher than of those
657 from wild-caught females. The study further revealed a selective retention of ARA and DHA
658 during early larval development reflecting important roles of these fatty acids, e.g. in
659 structural development, especially neural and visual functions (Sargent et al., 1999b). This is
660 similar to other studies on Florida pompano, *Trachinotus carolinus*, and common snook,
661 *Centropomus undecimalis* (Hauville et al., 2016), as well as Atlantic bluefin tuna, *Thunnus*
662 *thynnus* (Morais et al., 2011) where relative levels of ARA and DHA increased during the
663 first 4-6 dph together with utilization of total lipids as an energy source. The effect of EFA
664 levels on larval survival and development appears to be highly species specific. While a
665 positive effect of DHA is widely distributed (Glencross, 2009), the effect of ARA reaches
666 from positive (Mazorra et al., 2003), over neutral (Hauville et al., 2016) to negative (Røjbek
667 et al., 2014). In the Atlantic halibut, larval survival was found to be significantly higher from

668 females fed with an ARA enhanced diet (Mazorra et al., 2003), which coincides with our
669 results. In the European eel, overall, PUFAs were preserved in the larvae of the two farmed
670 as well as the wild caught groups, while mainly saturated fatty acids and MUFAs were used
671 during early larval development.

672 The most prominent difference between the wild-caught and farm-raised broodstock
673 was differences in embryonic survival and hatch success. The lower survival of offspring
674 from farm-raised females was related to a higher percentage of cleavage abnormalities
675 assessed at 4 hpf. Abnormal cleavage patterns have been shown to cause higher embryonic
676 mortality in Atlantic cod (Avery et al., 2009), yellowtail flounder, *Limanda ferruginea*
677 (Avery and Brown, 2005), and turbot, *Scophthalmus maximus* (Kjørsvik et al., 2003). Also in
678 the present case, the abnormal cleavages impeded embryonic development leading to a sharp
679 decline in survival between 8 hpf and 16 hpf, resulting in low hatch success.

680 This suggest that zygotic and early embryonic development in European eel subjected
681 to assisted reproduction is influenced by more factors than maternal nutrition and resulting
682 egg dry weight, lipid content and fatty acid composition (Mylonas et al. 2010). Here, an
683 important step in embryonic development is characterized by the maternal to zygotic
684 transition (MZT), in which developmental control is taken over by the activation of zygotic
685 transcription (Newport and Kirschner, 1982). This change takes place during the mid-blastula
686 transition, which in European eel occurs at ~10 hpf at 18°C (Sørensen et al., 2016b). Until
687 this point, maternal gene products are the most essential drivers for early embryonic
688 development. Studies have shown essential impacts of the abundance of specific mRNA
689 transcripts on egg quality and embryonic development (Aegerter et al., 2004; Lanes et al.,
690 2013; Rozenfeld et al., 2016; Škugor et al., 2014). The observed decline in survival of
691 embryos from farm-raised females around this time in embryonic development indicates
692 possible failure of the embryonic transcription as suggested by a previous study (Rozenfeld et

al., 2016). Further research should focus on detecting causes of the here revealed bottleneck of farm-raised eels throughout embryonic development in order to develop sustainable aquaculture for European eel.

Generally, wild-caught females produce gametes and offspring of higher quality including higher fertilization capacity of eggs and larval survival, exemplified by Atlantic cod (Lanes et al., 2012; Salze et al., 2005) and common sole, *Solea solea* (Lund et al., 2008). A possible explanation why wild-caught females might respond better to assisted reproduction procedures and produce eggs and offspring of higher quality may include differences in the endocrinological state of the females at the time of onset of therapy. This is also the background for feminization of eel that are later selected for broodstock. Here, estradiol is provided in the feed of juvenile eels for a period time to synchronize ovarian development in Japanese eel (Okamura et al., 2014; Tanaka, 2015). Likewise, the progress of early oocyte development and silvering-related changes may be stimulated by administration of androgens, e.g. 11-ketotestosterone (Di Biase et al., 2017; Lokman et al., 2015; Mordenti et al., 2018; Sudo et al., 2012). Such studies show that the androgen-pretreatment can enhance responsiveness to gonadotropic treatment, yet future research is needed to clarify, if such treatment would decrease embryonic development failure in farm-raised fish. Benchmarking the nutritional aspects, our results show that by modifying EFA content in the broodstock diet of farm-raised eels, nutritional egg quality parameters and fertilization rates comparable to wild-caught eels could be achieved. Particularly, larval survival was comparable and larval body area from farm-raised females fed Diet 1 for prolonged feeding was significantly higher than that of wild-caught females. These results indicate that once embryos undergo the MZT successfully and develop to completion, resulting larvae from farm-raised females fed enhanced diets are viable and of high quality up to the first feeding stage.

5 Conclusion

The enhanced diet composition and feeding regimes in the present study improved egg quality and offspring performance originating from farm-raised female European eel broodstock. By following embryonic and larval development, we documented the importance of egg dry weight, lipid content, and EFA during early ontogeny. Thus, the applied dietary levels of ARA, EPA and DHA enhanced the egg quality and offspring performance significantly in farm-raised broodstock highlighting, in particular, the importance of ARA. The results furthermore emphasized the importance of a long feeding duration in eel prior to onset of assisted reproduction. Not least, the dietary EFA levels combined with slow growth proved superior enhancing effects on egg and offspring quality. Additionally, comparing offspring quality between farm-raised and wild-caught broodstock in European eel revealed that the primary bottleneck in farm-raised offspring is during early embryonic development, where survival decreased significantly after 8 hpf. Thus, several factors besides nutritional factors may influence farm-raised broodstock performance and cause inferior embryonic development competence, e.g. the endocrinological state of the females at the onset of the induced maturation. Once hatched, however, larval survival was comparable between farm-raised females reared on the high ARA diet for a prolonged period and wild-caught broodstock. Notably, ARA and DHA were retained in the yolk sac larvae signifying their importance during early ontogeny. Together, findings of this study can be used in future experimental work to ascertain high offspring quality from farm-raised broodstock aiming at a closed cycle production in captivity.

Acknowledgements

Maria K. Johnsen, Elisa Benini, Dr. Sebastian N. Politis (Technical University of Denmark), and Dr. Sune Riis Sørensen (Billund Aquaculture) took part in the experimental work. Inge Holmberg, Inger Hornum, and Dr. Sigrun Jonasdottir (Technical University of Denmark) supervised laboratory work.

Funding

This study was part of the projects: Eel Hatchery Technology for a Sustainable Aquaculture (EEL-HATCH) and Improve Technology and Scale-up production of offspring for European eel aquaculture (ITS-EEL) supported financially by Innovation Fund Denmark, Grant no. 5184-00093B and 7076-00125B, respectively. Ian A.E. Butts was partially supported by the Alabama Agricultural Experimental Station and the USDA National Institute of Food and Agriculture, Hatch project (1013854). The funders provided support in the form of salary for the authors [JK, IAEB, JGS, IL, LH, CJ, JT], but did not have any additional role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supporting information

Table A.1. Number of females entering the analysis for each figure, figure part, treatment and sampling point of European eel, *Anguilla anguilla*.

Table A.2. Fatty acid composition (% of total fatty acids) of total lipids extracted from production 1 and 2 of the three experimental diets that were fed to European eel, *Anguilla anguilla* broodstock.

Table A.3. Fatty acid composition (% of total fatty acids) of total lipids extracted from unfertilized eggs of European eel, *Anguilla anguilla*, of farm-raised origin fed with Diet 1, Diet 2 or Diet 3 for either Trial 1 or Trial 2 or of wild-caught origin.

Table A.4. Fatty acid composition (% of total fatty acids) of total lipids extracted from larvae at 0 and 5 days post hatch of European eel, *Anguilla anguilla*, of farm-raised origin fed with Diet 1 (Trial 1 and Trial 2) or of wild-caught origin.

Figure Captions

Fig. 1 Total lipid content in eggs and larvae of European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A) and feeding trial (B) on total lipid (TL) in % dry weight (DW) of unfertilized eggs from farm-raised eels (n = 34). Effects of female broodstock origin (Diet 1 Trial 1 and Trial 2; wild-caught fish) (C), offspring age (Diet 1 Trial 1 and Trial 2; wild-caught fish) on TL of unfertilized eggs and larvae at 0 and 5 days post hatch (dph; D) (n = 24). Values represent means (± SEM) among females at each sampling point and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

Fig. 2. Relative fatty acid content in unfertilized eggs and larvae of European eel, *Anguilla anguilla*. Effects of maternal dietary regime and feeding trial on unfertilized egg levels (%) of ARA (A, B), EPA (C, D) and DHA (E, F) (n = 33). Effects of broodstock origin and age on eggs and larvae (Diet 1 Trial 1 and Trial 2; wild-caught fish) for ARA (G, H), EPA (I, J), and DHA (K, L). (n = 25). Values represent means (± SEM) among females at each age and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

Fig. 3. Fertilization success in European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A), and feeding trial (B) of farm-raised females (n = 23) as well as broodstock origin on fertilization success (C) (n = 19). Values represent means (± SEM) among females at each sampling time and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

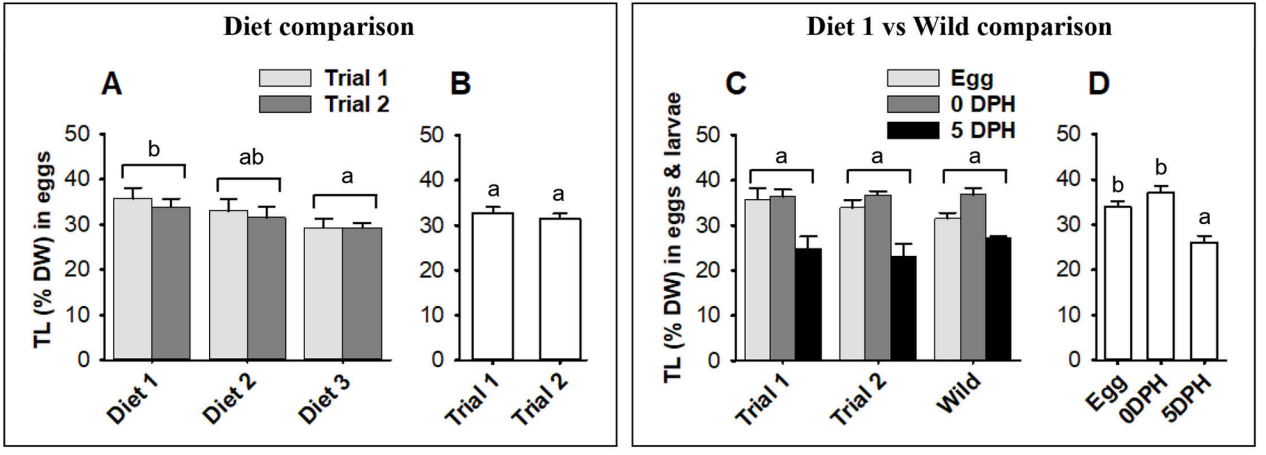
Fig. 4. Embryonic survival in European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A), feeding trial (B), and offspring age (4-48 hours post fertilization; C) on embryonic survival as well as their survival over time for individual females fed Diet 1 (D), Diet 2 (E), and Diet 3 (F) (n = 26). Effects of broodstock origin (G) and offspring age (H) on embryonic survival for individual females fed Diet 1 Trial 1 (I), Diet 1 Trial 2 (J) and wild-caught females (K) (n = 18). Values for bar plots represent means (± SEM) among females at each age and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

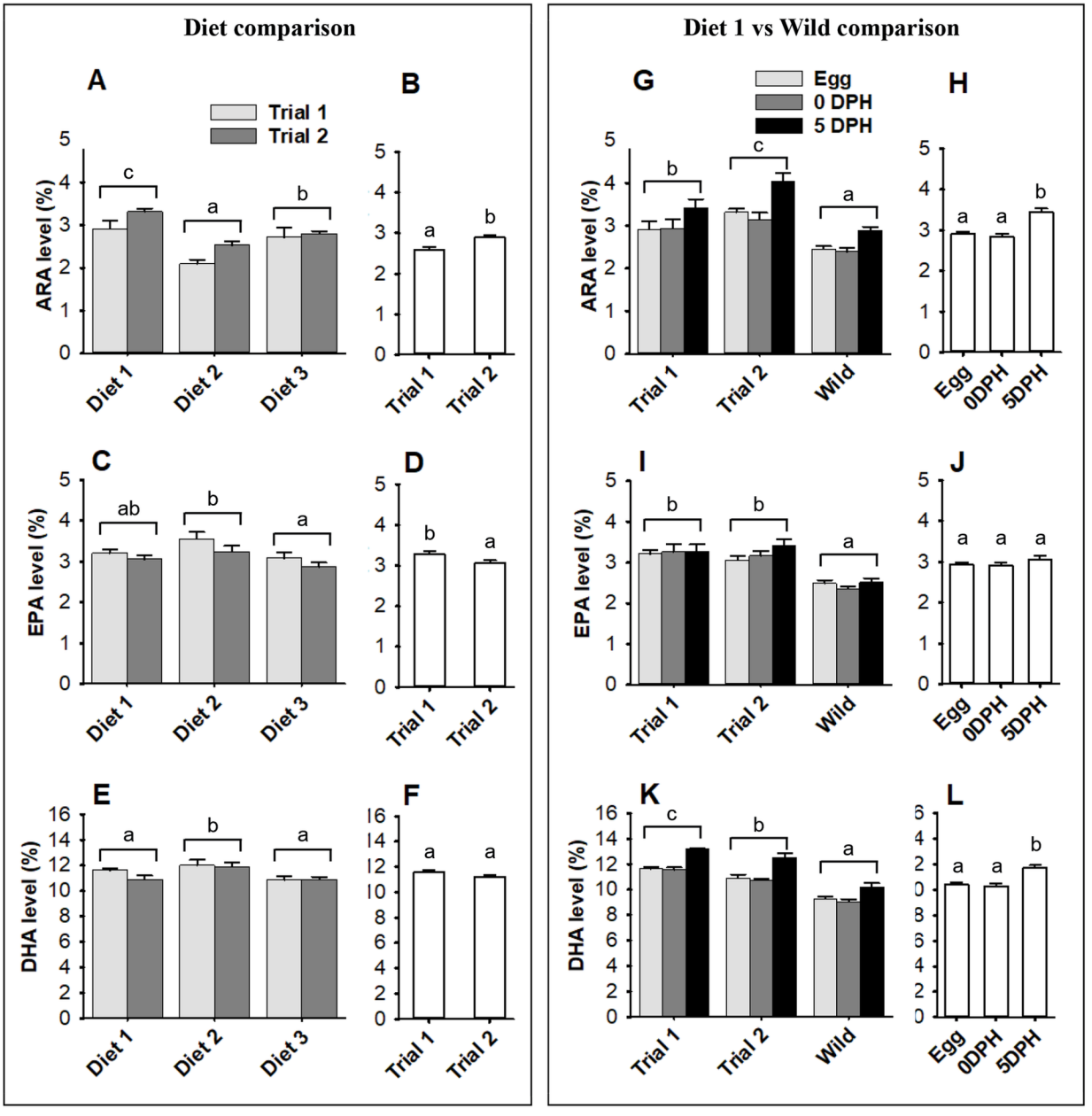
Fig. 5 Cleavage abnormalities in European eel, *Anguilla anguilla*. Normal (upper left) as well as typical abnormal cleavage patterns (A), effects of maternal dietary regime (B) and feeding trial (C) in farm-raised female eels (n = 22) as well as broodstock origin (D) (n = 19) on proportion of cleavage abnormalities in offspring at 4 hours post fertilization (hpf). Relationships between cleavage abnormalities at 4 hpf and embryonic survival at 48 hpf display offspring of individual females for all three treatments pooled (E), as well as individual treatments, Diet 1 Trial 1 (F), Diet 1 Trial 2 (G), wild-caught (H). Values for bar

plots represent means (± SEM) among female offspring at each age and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

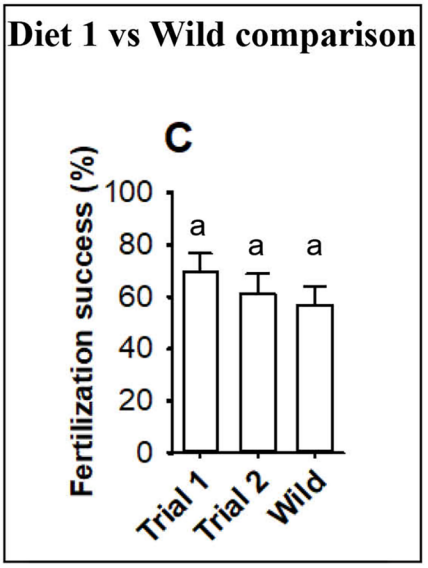
Fig. 6. Hatch success in European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A), feeding trial (B) (n = 24), and broodstock origin (C) (n = 18) on hatch success (%). Values represent means (± SEM) among females at each sampling point and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

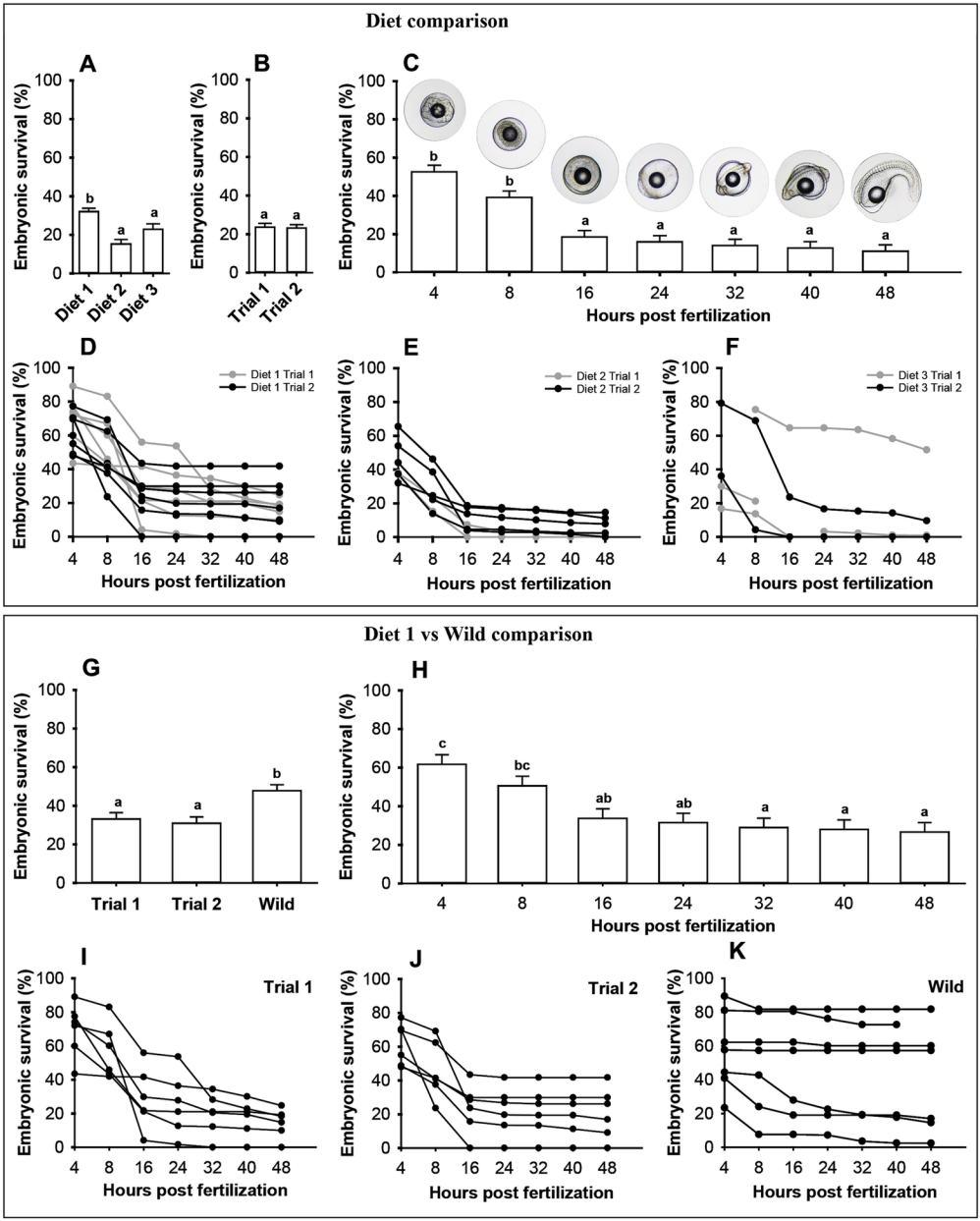
Fig. 7. Larval survival and development in European eel, *Anguilla anguilla*. Larval development throughout yolk sac stage (A), effects of broodstock origin (B) and offspring age (days post hatch, dph; C) on larval survival (n = 14). Survival of larvae from individual females in relation to maternal origin is displayed; Diet 1 Trial 1 (D), Diet 1 Trial 2 (E), and wild-caught females (F). Effects of broodstock origin and offspring age on standard length (mm; G, H), body area (mm^2; I, J), and oil droplet area (mm^2; K, L) (n = 13). Values for bar plots represent means (± SEM) among females at each age and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

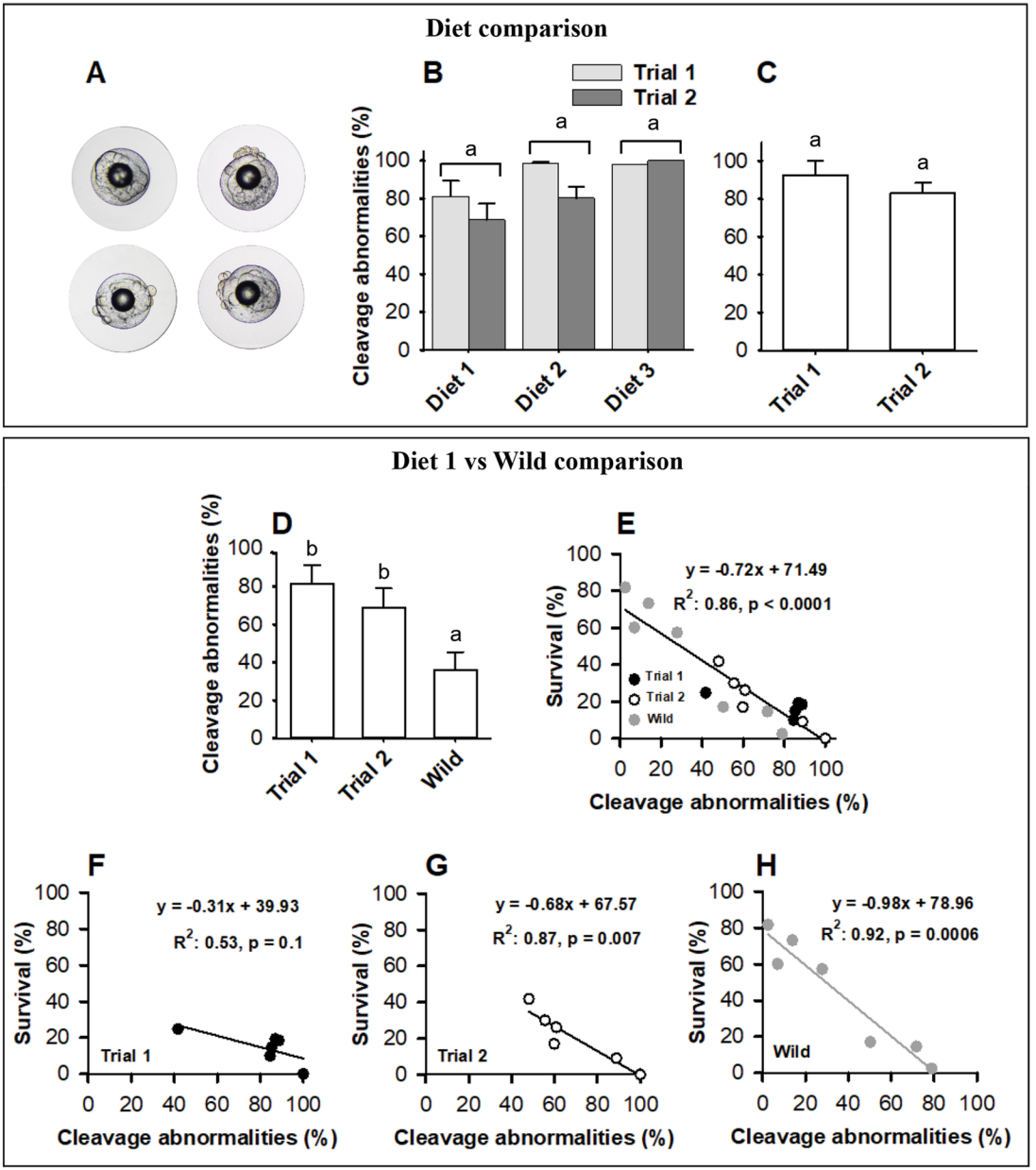

Diet comparison
A
Trial 1
Trial 2
B
TL (% DW) in eggs
50
40
30
20
10
0
b
ab
a
a
a
Diet 1
Diet 2
Diet 3
Trial 1
Trial 2
Diet 1 vs Wild comparison
C
Egg
0 DPH
5 DPH
D
TL (% DW) in eggs & larvae
a
a
a
b
b
a
Trial 1
Trial 2
Wild
Egg
0DPH
5DPH

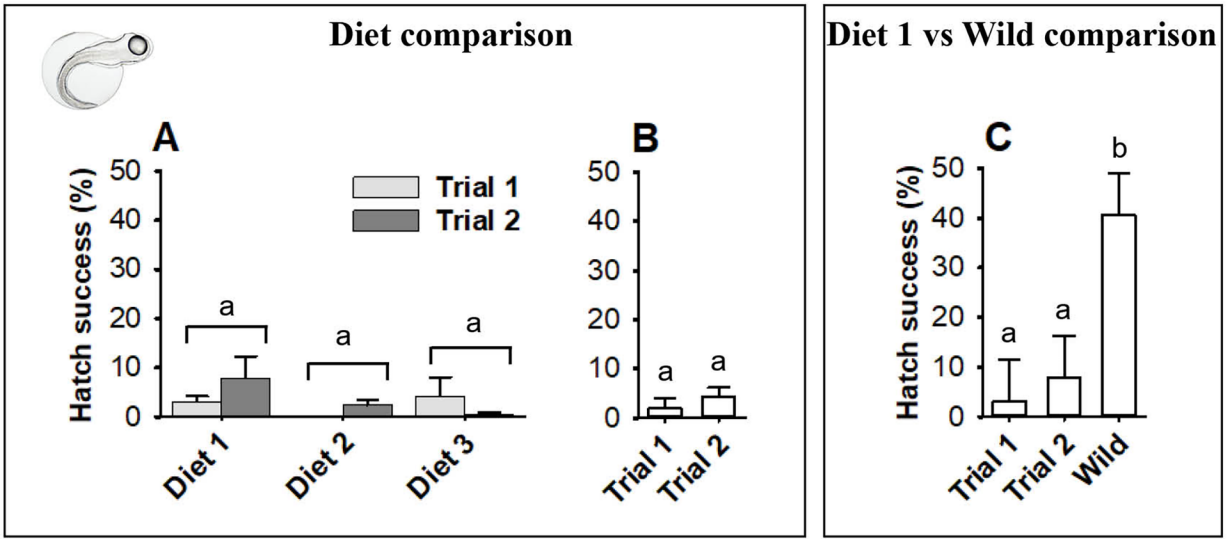

Diet comparison
Diet 1 vs Wild comparison
A
B
C
D
E
F
G
H
I
J
K
L
Trial 1
Trial 2
Egg
0 DPH
5 DPH
ARA level (%)
EPA level (%)
DHA level (%)
Diet 1
Diet 2
Diet 3
Trial 1
Trial 2
Wild
Egg
0DPH
5DPH

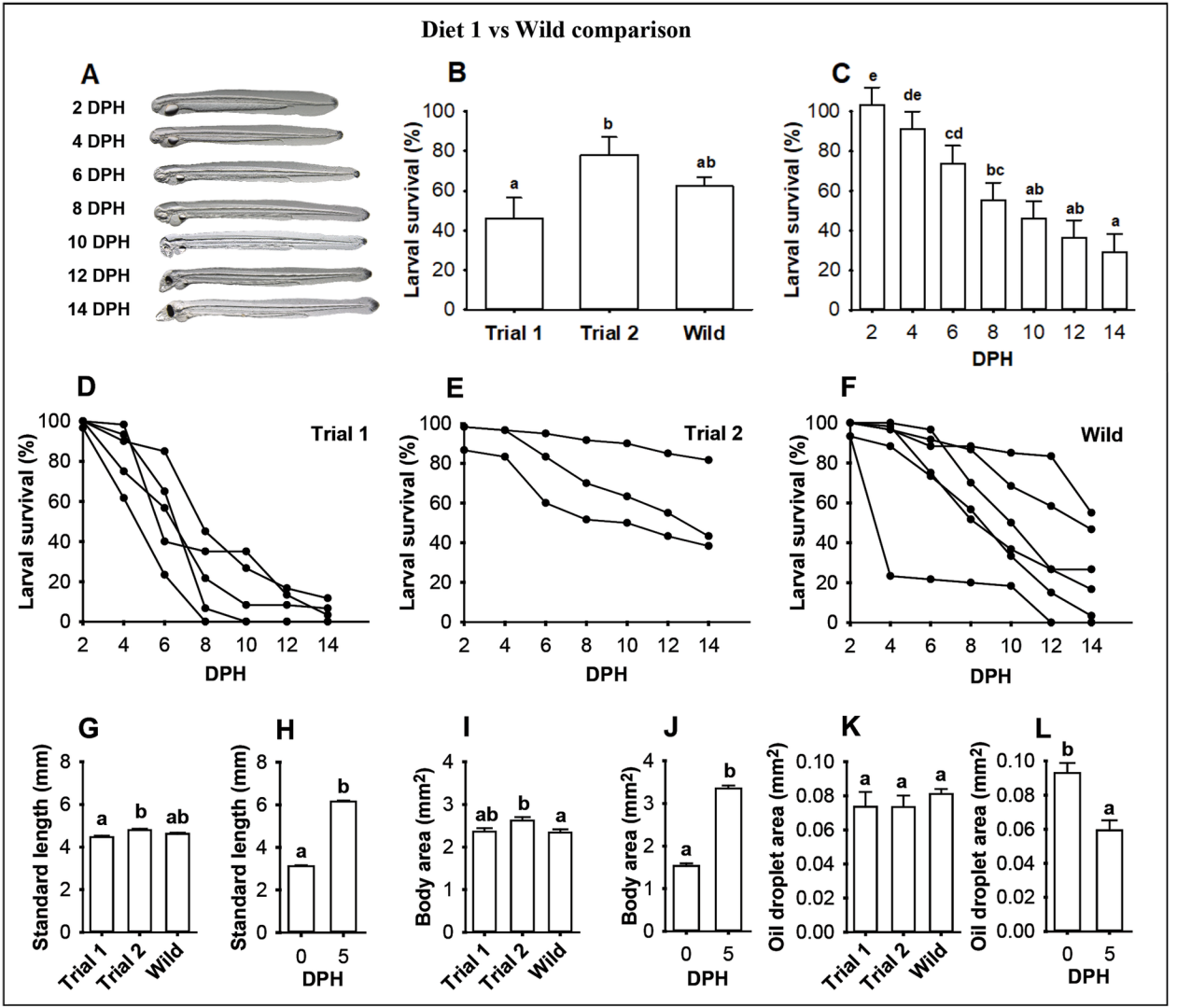
Diet comparison
A
Trial 1
Trial 2
Fertilization success (%)
100
80
60
40
20
0
b
a
a
Diet 1
Diet 2
Diet 3
B
a
a
Trial 1
Trial 2


Diet 1 vs Wild comparison
C
Fertilization success (%)
100
80
60
40
20
0
a
a
a
Trial 1
Trial 2
Wild


Diet comparison
A
B
C
Embryonic survival (%)
Diet 1
Diet 2
Diet 3
Trial 1
Trial 2
Hours post fertilization
D
E
F
Diet 1 Trial 1
Diet 1 Trial 2
Diet 2 Trial 1
Diet 2 Trial 2
Diet 3 Trial 1
Diet 3 Trial 2
Diet 1 vs Wild comparison
G
H
Wild
I
J
K


Diet comparison
A
B
Trial 1
Trial 2
Cleavage abnormalities (%)
a
a
a
Diet 1
Diet 2
Diet 3
C
a
a
Trial 1
Trial 2
Diet 1 vs Wild comparison
D
Cleavage abnormalities (%)
b
b
a
Trial 1
Trial 2
Wild
E
y = -0.72x + 71.49
R2: 0.86, p < 0.0001
Survival (%)
Trial 1
Trial 2
Wild
Cleavage abnormalities (%)
F
y = -0.31x + 39.93
R2: 0.53, p = 0.1
Survival (%)
Trial 1
Cleavage abnormalities (%)
G
y = -0.68x + 67.57
R2: 0.87, p = 0.007
Survival (%)
Trial 2
Cleavage abnormalities (%)
H
y = -0.98x + 78.96
R2: 0.92, p = 0.0006
Survival (%)
Wild
Cleavage abnormalities (%)


Diet comparison
A
B
Hatch success (%)
50
40
30
20
10
0
Trial 1
Trial 2
a
a
a
a
a
Diet 1
Diet 2
Diet 3
Trial 1
Trial 2
Diet 1 vs Wild comparison
C
b
a
a
Trial 1
Trial 2
Wild


Diet 1 vs Wild comparison
A
2 DPH
4 DPH
6 DPH
8 DPH
10 DPH
12 DPH
14 DPH
B
Larval survival (%)
a
b
ab
Trial 1
Trial 2
Wild
C
Larval survival (%)
e
de
cd
bc
ab
ab
a
DPH
D
Trial 1
Larval survival (%)
DPH
E
Trial 2
Larval survival (%)
DPH
F
Wild
Larval survival (%)
DPH
G
Standard length (mm)
a
b
ab
Trial 1
Trial 2
Wild
H
Standard length (mm)
a
b
DPH
I
Body area (mm²)
ab
b
a
Trial 1
Trial 2
Wild
J
Body area (mm²)
a
b
DPH
K
Oil droplet area (mm²)
a
a
a
Trial 1
Trial 2
Wild
L
Oil droplet area (mm²)
b
a
DPH