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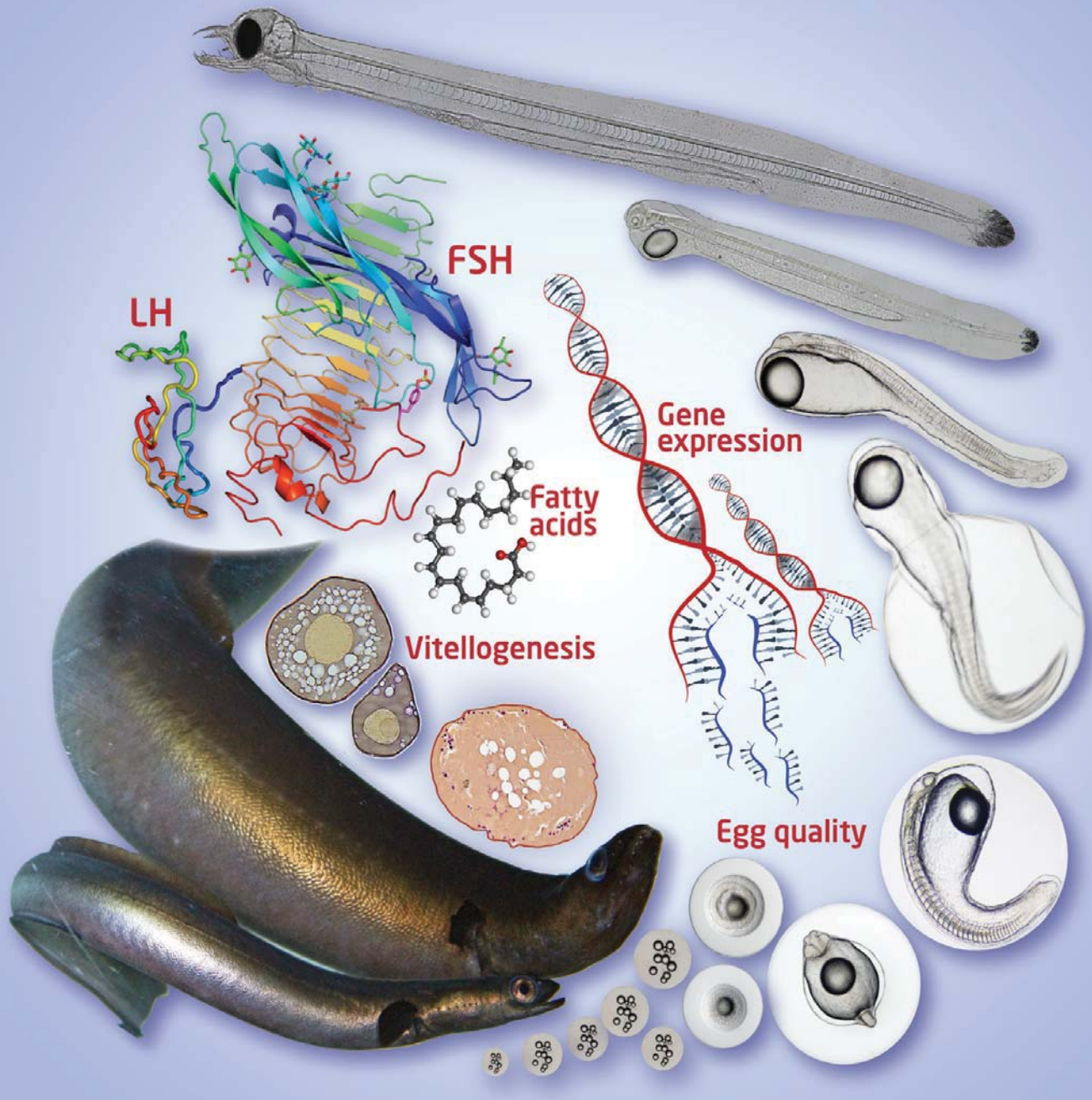
PhD Thesis



Egg Quality and Offspring Performance in European Eel

PhD Thesis

Johanna Sarah Kottmann

Submitted: February 7, 2020

Hirtshals, Denmark

Technical University of Denmark

National Institute of Aquatic Resources

Section for Marine Living Resources

Contents

List of abbreviations

11-kt: 11-ketotestosterone

ARA: arachidonic acid

CPE: carp pituitary extract

DHA: docosahexaenoic acid

DHP: 17α,20ß-dihydroxy-4-pregnen-3-one

dph: days post hatch

E2: estradiol-17β

EFA: essential fatty acid

EPA: eicosapentaenoic acid

FSH: follicle stimulating hormone

GSI: gonadosomatic index

GTH: gonadotropins

hpf: hours post fertilization

LC-PUFA: long-chain polyunsaturated fatty acids

LH: luteinizing hormone

MIS: maturation inducing steroid

mRNA: messenger RNA

MZT: maternal-to-zygotic transition

PE: pituitary extract

RAS: recirculating aquaculture system

SPE: salmon pituitary extract

T: testosterone

Preface

The present thesis was submitted as partial fulfilment of the requirements for obtaining the Doctor of Philosophy (PhD) degree. The thesis was performed at the Technical University of Denmark, National Institute of Aquatic Research (DTU Aqua) within the Fish Biology Research Group, Section for Marine Living Resources.

This PhD 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 the Innovation Fund Denmark, Grant no. 5184-00093B and 7076-00125B, respectively. The study was conducted between December 2015 and February 2020 and supervised by Dr. Jonna Tomkiewicz, main supervisor, and two co-supervisors Dr. Ian A.E. Butts, DTU Aqua/Auburn University (until July 2019, statistical analyses MS 1 and 2) and Prof. Helge Tveiten, UiT The Arctic University of Norway (from May 2019, steroid analyses MS 3). Experimental work was carried out at the EEL-HATCH facility, located at DTU Aqua in Hirtshals, Denmark, while the external research stay took place at UiT The Arctic University of Norway in Tromsø.

Prior to my enrollment as PhD student, I had valuable working experience in the EEL-HATCH project, employed as a Technical Assistant in Scandinavian Technical Marine Innovation (STMI), an industrial partner in the EEL-HATCH consortium. Through this, I was integrated into an international team with unique team-spirit and passion for eel reproduction research. During this time, I gained valuable hands-on-knowledge and insights into research on European eel as well as routine work in a hatchery facility. My fascination for eel research convinced me to pursue unanswered questions within eel captive reproduction and offspring quality and I was fortunate to qualify for a PhD fellowship. Concomitantly, I worked ¼ of a position as assisting facility manager at EEL-HATCH taking care of general broodstock husbandry and hatchery management.

During my PhD project, I performed three studies, each yielding a manuscript included in this thesis. In addition, I took part in a related fourth study led by PhD student, Michelle G.P. Jørgensen, and contributed as co-author to the publication (included in her PhD thesis). The three first authored studies focused on maternal impacts on egg quality and developmental competence in European eel. Here, the effect of maternal nutrition and assisted reproduction techniques on egg quality, embryonic as well as larval development have been assessed. This includes effects of fatty acid enhanced broodstock feeds, induced vitellogenesis using two different therapeutic agents (salmon and carp pituitary extracts), and steroid dynamics throughout embryogenesis following induced follicular maturation and strip spawning on egg and offspring quality. Sample analyses applied biochemical investigations, such as gas chromatography to identify and quantify fatty acid composition of lipids, radioimmunoassay to analyze steroid concentrations, and molecular tools, such as gene expression to understand underlying mechanisms of processes during

early development. Here, messenger RNA (mRNA) transcript patterns gave insights into the regulation of important development processes and provided first understanding of the underlying mechanisms of bottlenecks during early development of hatchery produced offspring, such as the maternal-to-zygotic transition. In this context, the studies of this thesis aimed at filling gaps in knowledge related to parameters affecting egg quality and offspring developmental competence in European eel to enhance viable larval production in particular from farm-raised broodstock getting one step closer to closing the life cycle in captivity.

The forth study, complementing the second study of this thesis, focused on comparing a constant vs. an increasing dose of carp pituitary extracts as agent inducing vitellogenesis, while closely following females during ovarian development and evaluating offspring quality. The study, mentioned in the introduction, enhanced molecular insights into endocrinology through expression of hormone receptors in the ovary and liver, as well as genes involved in growth, development, and stress response throughout vitellogenesis. My contribution related to the experimental work, scientific discussions, and developing the MS alongside Study 2 of this thesis.

- Jørgensen, M.G.P., **Kottmann, J.S.**, Miest, J.J., Dufour, S., Kjørsvik, E., Tomkiewicz J. Impact of carp pituitary extract at constant or increasing dose on ovarian development, expression of key genes and reproductive success in European eel, *Anguilla anguilla*. *Manuscript.*

Lastly, I contributed to a published book chapter that reviewed the progression and current status of research in eel hatchery technology performed at DTU by the Fish Biology Research Group in collaboration with industrial partners. My contributions related to female broodstock nutrition and assisted reproduction are referenced in this thesis.

- Tomkiewicz, J., Politis, S.N., Sørensen, S.R., Butts, I.A.E., **Kottmann, J.S.**, 2019. European eel – an integrated approach to establish eel hatchery technology in Denmark. In (Eds. Don, A., Coulson, P.) Eels - Biology, Monitoring, Management, Culture and Exploitation: Proceedings of the First International Eel Science Symposium. Sheffield: 5m Publishing. p. 340-374.

Hirtshals, 7th February 2020

Johanna S. Kottmann

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I would like to thank all colleagues from DTU Aqua for making my past few years so enjoyable and memorable. I was lucky to meet many people from all over the world during my PhD, forming memories that will never be forgotten: A huge and heartfelt thanks to all my friends in Hirtshals, all the time we spent together and the home Hirtshals has been for me during the past few years.

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English summary

Closing the life cycle of fish and shellfish in aquaculture requires a stable production of high quality gametes from farm-raised broodstock. However, the gamete quality of fish in captivity is often low or variable. In particular, egg quality is highly influenced by maternal factors, such as nutrition and reproduction techniques, which need to be addressed in broodstock management. To increase the production of high quality eggs intended for hatcheries, a better understanding of factors that regulate gamete and offspring quality is required. In the case of European eel, *Anguilla anguilla*, assisted reproduction in terms of hormonal manipulation is successfully applied. However, viable egg production is variable, affecting efficient egg and larval production. Here, maternally derived constituents of the egg, such as nutrients, messenger RNA (mRNA) transcripts and steroid hormones determine early developmental competence and survival of the embryos. The studies of the present PhD addressed maternal factors influencing egg quality and embryonic development in European eel using experimental trials and a combination of biochemical and molecular analysis tools. As such, this thesis includes three studies investigating the effect of i) broodstock nutrition, ii) hormonal induction of vitellogenesis, and iii) induced follicular maturation and steroid transfer, on egg quality and offspring developmental competence. In particular, focus is set on the embryonic developmental phase that represents the transition from maternal to embryonic control and may constitute a bottleneck in early life history.

A first study (Study 1) addressed the impact of female broodstock diets varying in essential fatty acid (EFA) composition and feeding regimes on egg fatty acid composition as well as the resulting embryonic and larval development. Here, feeding farm-raised broodstock with EFA enhanced diets increased the total lipid content of eggs, the amount of floating eggs, fertilization success, and embryonic survival. Moreover, prolonged feeding with further enhanced diets led to higher levels of one EFA (arachidonic acid) and dry-weight of eggs as well as improved larval survival. The study benchmarked offspring quality from farm-raised females with those of their wild-caught counterparts and showed significantly higher embryonic survival and lower occurrence of cleavage abnormalities in offspring obtained from wild-caught females. Here, the phase of maternal-to-zygotic transition (MZT) occurring during the mid-blastula transition represented a bottleneck during embryonic development, in particular for offspring from farm-raised females. However, once hatched, larval quality was comparable between farm-raised and wild-caught females for the best performing feed type.

Thereafter, two studies focused on assisted reproduction techniques in European eel. Here, Study 2 compared the effect of using either carp (CPE) or salmon pituitary extracts (SPE) on egg quality and embryonic development. Both therapeutic agents led to viable egg and larval production. However, using SPE to induce vitellogenesis resulted in higher amounts of floating eggs, higher embryonic survival, and lower occurrence of cleavage abnormalities. These differences were linked to differences in mRNA transcript abundance

for genes involved in cell adhesion, MZT activation, and immune regulation. The study observed differential expression patterns of 20 genes throughout embryonic development with 12 of these genes being associated with cleavage abnormalities, embryonic survival or hatch success.

Study 3 addressed the second step in assisted reproduction involving induction of follicular maturation, which is manifested in the resumption of meiosis and attainment of developmental competence of the eggs. Here, focus was on the transfer of sex steroids to the egg and related impact on offspring quality. Steroids were present in the unfertilized eggs, while concentrations subsequently decreased during embryogenesis. In particular, concentrations of estradiol-17β (E2) and 17α,20ß-dihydroxy-4-pregnen-3-one (DHP), i.e. the maturation inducing hormone, negatively impacted embryonic developmental competence in terms of fertilization success, embryonic survival, occurrence of cleavage abnormalities, and hatch success. Moreover, this study examined mRNA abundance patterns throughout embryogenesis of genes related to stress response, the somatotropic axis as well as thyroid and lipid metabolism. Here, mRNA transcripts were maternally transferred with the majority of genes showing increased expression after the MZT. The abundance of three genes involved in thyroid or lipid metabolism was related to embryonic developmental competence. Moreover, the concentrations of steroids (E2 and testosterone) in the unfertilized eggs were associated with the mRNA abundance of genes involved in thyroid metabolism.

In conclusion, this PhD project substantiated insights into maternal impacts on egg quality and resulting offspring performance in European eel by experimentally studying dietary EFA enhancement and two steps in assisted reproduction. Study 1 showed that improving EFA in broodstock diets led to improved embryonic survival in offspring of farm-raised females and enabled a production of larvae of similar quality as those of wild-caught females. Nonetheless, embryonic survival in offspring of wild-caught females was higher and appeared to be related to factors involved in the MZT. Hormonal treatments with different types of gonadotropin used (Study 2) impacted the offspring developmental competence in terms of embryonic survival and occurrence of cleavage abnormalities. Also, the maternal transfer of sex steroids (Study 3) likely partially derived from induced follicular maturation to the egg and lack of metabolic degradation throughout embryogenesis had a negative effect on the developmental potential. The study identified differential expression patterns of mRNA transcripts throughout embryogenesis with particular focus on the MZT. Prospectively, the thesis linked the bottleneck in survival to cleavage abnormalities and developmental failure during the period of MZT. As such, this PhD thesis elucidated important aspects of broodstock management and biomarkers of gamete quality that may be addressed in future research to enhance egg quality of this ecologically and economically important species in nature and aquaculture.

Dansk resumé

En stabil produktion af æg og sæd af høj kvalitet fra opdrættede stamfisk er nødvendig for at lukke fisks og skaldyrs livscyklus i akvakultur. Kvaliteten af kønsceller hos fisk i kultur er imidlertid ofte lav eller variabel. Særligt æggenes kvalitet påvirkes af forhold omkring moderfisken, såsom dens ernæring og reproduktionsteknik, hvilket må adresseres i opdræt og håndteringen af stamfisk. For at kunne øge produktionen af æg af høj kvalitet i klækkerier er det nødvendigt at forbedre forståelsen af de faktorer, der regulerer kvaliteten af både æg, fostre og larver. Her er æggets indhold af næringsstoffer, messenger-RNA (mRNA) og hormoner fra moderfisken væsentlige for fosterets udviklingsevne og overlevelse. For europæisk ål, *Anguilla anguilla*, er det muligt at producere levedygtigt afkom ved hjælp af assisteret reproduktion, dvs. hormonbehandling, hvilket imidlertid påvirker kvaliteten af æg og dermed en effektiv larveproduktion. Studierne i dette ph.d.-projekt undersøger effekten af flere faktorer, der kan påvirke ægkvaliteten og fosterudviklingen hos europæisk ål. Forskningen omfatter eksperimentelle forsøg kombineret med biokemiske and molekulære analysemetoder. Afhandlingen omfatter tre studier, der tilsammen undersøger effekten af i) moderfisks ernæring, ii) hormonel induktion af modning og iii) induceret slutmodning på ægkvaliteten og afkommets udviklingsevne. Fokus er på den embryonale udviklingsfase, hvor udviklingen går fra at være kontrolleret af moderfiskens mRNA integreret i ægget til aktivering af embryonets eget genom, hvilket kan være en flaskehals i den tidlige udvikling.

Det første studie undersøgte effekten af forskellige fodertyper og varigheden af fodringsperioden på kvaliteten af æg, herunder deres evne til at udvikle sig og senere blive til larver. Her var mængden og sammensætningen af essentielle fedtsyrer (EFA) i foderet sammenholdt med foderperiodens varighed og hunfiskenes vækst i centrum. Resultaterne viste, at foder beriget med EFA kan øge lipidindholdet i æggene, mængden af flydeæg, befrugtningssuccessen og fosteroverlevelsen hos afkom fra opdrætsfisk. Desuden førte en forlænget foderperiode med yderligere EFA-forbedret foder til større indhold af fedtsyren arachidonsyre og højere tørvægt hos æggene samt forbedret larveoverlevelse. Studiet sammenlignede også kvaliteten af afkom fra opdrætsfisk og vildfangede blankål. Resultaterne viste en signifikant lavere forekomst af irregulære celledelinger og højere overlevelse gennem fosterstadiet hos afkom fra vildfangede hunner. Her repræsenterede overgangen ”maternal-to-zygotisk transition” (MZT), der forekommer i mid-blastulastadiet, en flaskehals under forsterudviklingen, især for afkom fra opdrættede moderfisk. Efter at æggene var klækket, var kvaliteten af larver fra den bedste fodertype imidlertid sammenlignelig med larver fra vildfangne blankål, hvilket er lovende resultater.

De to øvrige studier omhandler hormonelle reproduktionsteknikker, der anvendes i forplantning af den europæiske ål i kultur. Studie 2 sammenlignede effekten af karpe- (CPE) og laksehypofyseekstrakt (SPE) på modning af hunfisk, ægkvalitet og embryonaludvikling. Begge metoder førte til levedygtige æg og larveproduktion. Resultaterne viste, at SPE-behandlede hunner gav flere flydeæg, færre uregelmæssige

kløvninger og højere fosteroverlevelse. Disse forskelle var knyttet til æggenes indhold af mRNA relateret til gener involveret i celleadhæsion, MZT-aktivering og immunregulering. I alt så undersøgelsen på gen-ekspressionsmønstre for 20 gener gennem forsterudviklingen, hvoraf 12 viste sig at være forbundet med irregulære celledelinger, fosteroverlevelse eller klækningssucces.

Studie 3 vedrører andet trin i den hormonelle reproduktionsteknik, som involverer induktion af slutmodningen. Dette trin manifesterer sig som genoptagelse af meiosen og igangsættelse af processer, der tilsammen gør, at ægget til slut opnår befrugtnings- og udviklingskompetence. Her viste analyser af blodprøver fra moderfisk, æg og fostre, at der blev overført kønshormoner især østradiol-17p (E2) og 17a, 20ß-dihydroxy-4-graven-3-on (DHP), dvs. det modningsinducerende hormon fra moderfisk til æg og fra æg til foster. Disse steroider var således til stede i de ubefrugtede æg, mens koncentrationerne faldt gennem embryogenesen. Her påvirkede høje niveauer af E2 og DHP, den embryonale udviklingsevne negativt med hensyn til både befrugtningssucces, fosteroverlevelse, forekomst af irregulære celledelinger og klækningssucces. Studiet undersøgte desuden forekomsten mRNA-for gener relateret til stressrespons, den somatotropiske akse såvel som thyreoidea- og lipidmetabolisme gennem embryogenesen. Her blev mRNA viste størstedelen af generne en stigende ekspression efter MZT. Kun tre af generne involveret i thyreoidea- eller lipidmetabolisme var relateret til fosterets udviklingsevne. Koncentrationerne af steroider (E2 og testosteron) i de ubefrugtede æg viste sig endvidere at være forbundet med mRNA-mængden af gener involveret i thyreoideametabolisme.

Ved eksperimentelt at studere foderforbedring og hormonale reproduktionsteknikker bidrager dette ph.d.-projekt i sin helhed med ny indsigt i moderfisks påvirkning af ægkvalitet og resulterende kvalitet af fostre og larver hos europæisk ål. Studie 1 viste, at forbedring af EFA i foder til moderfisk medførte forbedret overlevelse hos afkom fra opdrætsål, selvom den var lavere end for vildfangede hunål. Imidlertid var larver fra det bedste foder af samme kvalitet som hos vildfangede hunner. Hormonbehandlinger med to hyppigt anvendte typer af gonadotropin i studie 2 viste forskelle i effekt på afkommets udviklingsevne med hensyn til forekomst af irregulære kløvninger og fosteroverlevelse. Dertil kommer undersøgelserne i studie 3 en overførsel kønshormoner til ægget, hvilken sandsynligvis hænger sammen med den SPE- og DPH-inducerede slutmodning. Her viste resultaterne, at manglende metabolisk nedbrydning af hormonerne hos fosteret gennem hele udviklingsforløbet havde en negativ effekt på udviklingspotentialet. Studie 3 identificerede desuden forskelle i genekspressionsmønstre gennem hele embryogenesen med særlig fokus på stigning efter MZT. Samlet peger studierne på en flaskehals i fosteroverlevelsen grundet fejl i celledelinger og blastuladannelse samt aktiveringen af MZT, antagelig relateret til reproduktionsteknikker, mens foderforbedring kan skabe en høj kvalitet larver. Afhandlingen belyser derigennem væsentlige aspekter i opdræt af stamfisk med en indikation af biomarkører for gamet- og ægkvalitet, hvilket kan fremme forskning og udvikling både generelt for fisk i akvakultur og specifikt inden for ressourceeffektivt opdræt af ål.

Synthesis

1. Aquaculture and hatchery technology

The development of the aquaculture sector over the past decades is remarkable and it continues to grow faster than any other food production sector (FAO, 2018). In 2016, about 88 % of the total fish production was directly used for human consumption. The total number of cultured aquatic species worldwide has increased by 26.7 % over the past decade to a present diversity of ~600 species (FAO, 2018). Worldwide, the contribution of aquaculture to total seafood production has increased to 46.8 %. In Europe, however, the aquacultural share only accounts for 18 % illustrating a potentially unexploited market share to comply with the increasing demand for seafood production (FAO, 2018). The growing importance and dynamic development of aquaculture depends on its sustainability. Here, land-based aquaculture in form of Recirculating Aquaculture Systems (RAS) are expected to be the key instrument for future aquaculture, providing a consistent and reliable source of high quality seafood in an environmentally sustainable way (Ebeling and Timmons, 2012). RAS technology has the main advantages of using less water, having a controllable rearing environment, and stable biophysical conditions (temperature, salinity, water quality parameters, such as, dissolved oxygen, carbon dioxide, ammonia, nitrite, nitrate, pH, and suspended solids) (Dalsgaard et al., 2013; Ebeling and Timmons, 2012). Moreover, targeted aquaculture allows for diversification of culturable species providing an increased supply of diverse fish products. Species diversification is of high importance for long-term production and improving the development of the auquaculture industry towards more sustainable production – both on a global and regional level (Metian et al., 2019). This includes domestication, which describes the process of modifying animals from their wild ancestors through selective breeding in captivity, making it useful for human consumption (Teletchea, 2015; Teletchea and Fontaine, 2014). Here, closing the life cycle is the prerequisite for domestication of targeted species focussing on developing hatchery technology and facilitating breeding programmes allowing year-round hatchery production. Together, this allows the replacement of capture-based aqauculture therefore releasing pressure on wild-stocks (COM, 2013; STECF, 2014; Teletchea, 2015).

1.1. Gamete quality and broodstock management

Closed-cycle aquaculture production relies on the production of high quality gametes producing viable offspring with selected traits needed for farming (Migaud et al., 2013; Mylonas et al., 2010). To date, the year-round production of high quality eggs is one of the major challenges in the aquaculture industry. Basic and applied research is needed on all aspects of broodstock management practices to ensure optimal conditions for obtaining stable high quality broodstock in captivity. Here, common factors of broodstock husbandry that influence gamete quality include nutrition, environmental conditions such as temperature, photoperiod and general culture conditions, stress, age of the fish, and

spawning induction procedures (Bobe and Labbé, 2010; Migaud et al., 2013). Addressing these challenges is vital for the sustainable development of future aquaculture industry.

1.1.1. Broodstock nutrition

Broodstock nutrition has been outlined as one of the essential factors for a successful aquaculture production and has attracted a great deal of attention in teleost research (Izquierdo et al., 2001). Improving broodstock nutrition has been shown to enhance reproductive success and offspring quality in many teleost species. Here, nutrients, such as lipids, proteins, carbohydrates, vitamins, and minerals are incorporated into the egg prior to and/or during vitellogenesis to enhance egg and sperm quality and ensure successful development of the offspring. If the content of any of these components is not matching the embryos requirements, the somatic development risks being compromised (Brooks et al., 1997; Izquierdo et al., 2001; Lubzens et al., 2010). Within nutritional demands, lipids and particularly their constituent fatty acids and mutual ratios range among major factors influencing reproductive success and early life history in teleosts (Izquierdo et al., 2001). Lipids are hydrophobic small molecules that, together with proteins, represent the major organic constituent of fish (Tocher, 2003). Their essential functions include i) metabolic energy in form of ATP via mitochondrial β-oxidation; ii) structural components of cell membranes; iii) precursors of eicosanoid production (Glencross, 2009; Sargent et al., 1995; 2002; Tocher, 2003). Lipids have been classified into eight groups: fatty acids; glycerolipids; glycerophospholipids; sphingolipids; sterol lipids; prenollipids; saccharolipids; and polyketides (Fahy et al., 2009). With respect to fatty acids, their function and importance has been extensively studied. Particularly, n-3 and n-6 fatty acids have received attention, i.e. long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid (DHA; 22:6n-3), eicosapentaenoic acid (EPA; 20:5n-3), and arachidonic acid (ARA; 20:4n-6), which are characterized by ≥ 20 carbon atoms and ≥ 3 bonds. Here, DHA, EPA, and ARA as well as their mutual ratios have been shown to impact early development of many teleost species (Izquierdo et al., 2001). Marine teleosts have, however, only limited capacity to synthesize LC-PUFA and they therefore rely on the dietary intake of these essential fatty acids (EFA). Thus, female broodstock diets must include the required LC-PUFA for the production of high quality eggs and healthy larvae.

1.1.2. Captive reproduction

The capability to fully control the reproductive processes in captivity is an essential step for the year-round production of high quality gametes. The reproductive cycle of fish consists of two main phases: i) gamete growth and development (spermatogenesis and vitellogenesis) and ii) spermiation and follicular maturation (Mylonas et al., 2010).

Fish oogenesis commences with the formation of primordial germ-cells (PGCs), followed by their transformation into oogonia and primary growth oocytes (Lubzens et al., 2010). During the reproductive phase, previtellogenic oocytes transform into vitellogenic stages that are characterized by a period of extensive growth mainly through build-up of

nutritional reserves within their cytoplasm covering the embryos needs. This occurs through vitellogenin synthesis in the liver and their processing into yolk proteins in the oocyte but also includes other molecules, such as lipids and vitamins. Furthermore, in pelagophil species, defined by producing buoyant eggs, often accumulate lipid droplets that gradually fuse together to produce one larger lipid globule, adding to the positive buoyancy of the egg and early embryos (Cerdà et al., 2007). The oocyte also accumulates maternal messenger RNA (mRNA) and other constituents, thereby influencing gamete quality and early life history (Brooks et al., 1997; Lubzens et al., 2010). By the end of the vitellogenic period, the follicular maturation process starts with migration of the germinal vesicle (GVM) towards the animal pole and resumption of meiosis (completion of the first meiotic division followed by progression to metaphase II). Further processes involve breakdown of the germinal vesicle breakdown (GVBD), chromosome condensation, assembly of the meiotic spindle, and formation of the first polar body (Nagahama and Yamashita, 2008). The female gamete has now become a haploid ovum. These processes are required for subsequent successful fertilization. At the end of the maturation process, ovulation occurs, where the matured oocyte is being released from the surrounding follicular cell layers into the ovarian cavity (cystovarian type) or the abdominal cavity (gymnovarian type) depending on the species (Kagawa, 2013). The cystovarian type occurs in many teleost fishes, while the gymnovarian type is characteristic of salmonids and eels. During fertilization, the female and male gametes fuse forming the diploid egg (Lubzens et al., 2010).

In teleosts, vitellogenesis and follicular maturation are primarily regulated by i) gonadotropins (GTH), follicle stimulating hormone (FSH) and luteinizing hormone (LH), ii) maturation-inducing steroid (MIS), and iii) maturation-promoting factor (MPF) (Nagahama and Yamashita, 2008). Release of the pituitary GTHs, FSH and LH, is regulated by the brain, stimulating the synthetic agonists of gonadotropin-releasing hormone (GnRH). After being released into the bloodstream, LH and FSH stimulate the production of sex steroids such as estrogens (e.g. estradiol-17β, E2), androgens (e.g. testosterone, T), 11-ketotestosterone,11-kt), and progestogens (e.g. 17α,20ß-dihydroxy-4-pregnen-3-one, DHP) in ovarian follicles. Here, FSH appears to have a more pronounced role during vitellogenic growth of follicles, partly through stimulation of E2, which is commonly known for the synthesis of the yolk precursor vitellogenin in the liver. Moreover, androgens, though considered male hormones, are involved in oocyte growth and lipid accumulation in pre-vitellogenic and vitellogenic stages. LH is known to be involved in the follicular maturation through stimulation of MIS (Nagahama and Yamashita, 2008). Among progestogens, DHP is the most effective steroid in most teleost species and is essential for the induction of oocyte maturation (Nagahama, 1983; Nagahama and Yamashita, 2008). The essential role of steroids during different phases of the oogenesis is widely accepted (Kazeto et al., 2011; Tokarz et al., 2015; Yaron et al., 2003).

Finding the optimal conditions for broodstock to reproduce in captivity is an important factor of broodstock management. In some aquaculture species, reproduction in captivity

proceeds naturally and can be managed by adjusting rearing conditions, such as water temperature, photoperiod, or spawning substrate (Mylonas et al., 2010). In other species, reproduction needs to be controlled by hormonal induction of gametogenesis or gamete maturation. Here, product type and treatment schemes are species-specific. However, the application of hormonal treatments for reproduction of fish in aquaculture may impact reproductive success, egg quality, and early life history (Mylonas et al., 2010).

1.2. Biomarkers of gamete quality

Egg quality is mostly defined as the ability of an egg to be fertilized and produce viable offspring (Bobe and Labbé, 2010). Thus, the evaluation of egg quality requires monitoring both, prior to and after fertilization to identify biomarkers. Predictive markers for gamete quality have important applications in research, e.g. optimizing hatchery protocols, and may be beneficial in identifying poor quality gametes in the aquaculture industry (Migaud et al., 2013). However, biomarkers appear to be highly species-specific. Size, dry-weight, and morphology of unfertilized eggs may be used in some species to evaluate the developmental potential, but results appear inconsistent and have limitations (Bobe and Labbé, 2010). After fertilization, the most common ways to evaluate the developmental potential include proportion of floating eggs, fertilization success, embryonic survival at key steps, and hatch success (Bobe and Labbé, 2010). In marine species with pelagic eggs, the proportion of floating eggs after fertilization by nature indicates egg quality. Furthermore, such pelagic eggs are generally transparent, making division of blastomeres from the zygote visible. Here, the symmetry of blastomeres may provide a valuable tool to assess the developmental competence of the egg (Bobe and Labbé, 2010; Brooks et al., 1997; Kjørsvik et al., 1990). Over the past decades, increasing attention has been given to molecular mechanisms that may define egg quality (Bobe and Labbé, 2010; Lubzens et al., 2017; Sullivan et al., 2015). Undoubtedly, the quality of an egg is highly affected by components incorporated during vitellogenesis, such as lipids, maternal mRNA, and hormones (e.g. sex steroids) and thus transferred to the offspring (Brooks et al., 1997; Lubzens et al., 2010; Tokarz et al., 2015). As such, maternally inherited mRNA transcripts influence early embryonic development until the developmental control is taken over by the embryo through activation of the zygotic transcription, which occurs during the mid-blastula transition (Newport and Kirschner, 1982). Schematics on the progress of the MZT during embryonic development are presented in Figure 1. Here, it needs to be considered that the patterns likely are more progressive than originally thought and activation as well as degradation take place gradually during different phases (Mathavan et al., 2005; Traverso et al., 2012). Nonetheless, the successful process and timing of this transition, including clearance of maternal mRNA and activation of zygotic transcription appear to be crucial for embryonic development (Giraldez et al., 2006; Lee et al., 2014). The abundance of specific maternally inherited mRNA transcripts has been related to egg quality and embryonic development in various teleost species (Aegerter et al., 2004; Lanes et al., 2013; Mommens et al., 2010; Rozenfeld et al., 2016; Škugor et al., 2014; Traverso et al.,

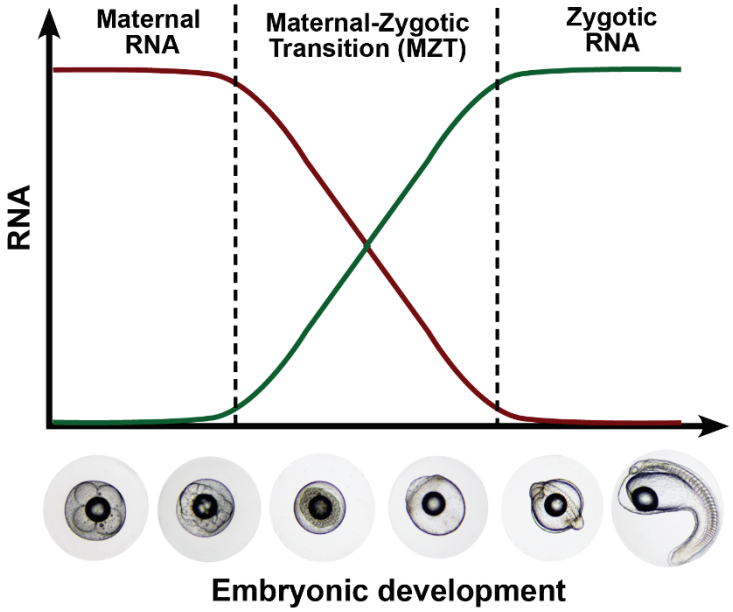


Figure 1. Schematics of the maternal-to-zygotic transition in marine fish. Pictures represent embryonic developmental stages of the European eel, *Anguilla anguilla.*

2012). This may provide valuable new insights, important to our understanding of the molecular mechanisms regulating egg quality.

Another aspect concerns the hormonal content of the egg and their effects on the developmental potential. To date, insights into the natural development are limited, even in a model organism such as the zebrafish, *Danio rerio* (Bobe and Labbé, 2010; Tokarz et al., 2013). As the activation of the hypothalamus–pituitary–interrenal (HPI) axis and *de novo* steroid synthesis in teleosts is likely not initiated until after hatch, it is suspected that maternally derived steroids affect early life history of fish (Nesan and Vijayan, 2013). Steroids of maternal origin (among others T, E2, 11-kt, and DHP) have been measured in unfertilized eggs of Coho Salmon, *Oncorhynchus kisutch* (Feist et al., 1990), Arctic charr, *Salvelinus alpinus* (Khan et al., 1997), and threespined stickleback, *Gasterosteus aculeatus* (Paitz et al., 2015). Concentrations showed a steady decrease throughout embryonic development indicating metabolization by the embryo. Moreover, higher concentrations of DHP and E2 were found in non-viable eggs than in viable eggs in Coho salmon (Feist et al., 1990). The presence of cortisol has also been reported in various species, e.g. zebrafish (Alsop and Vijayan, 2008), tilapia, *Oreochromis mossambicus*, (Hwang et al., 1992), Japanese flounder, *Paralichthys olivaceus* (de Jesus et al., 1991) and maternal exposure to cortisol led to detrimental effects on the offspring, such as increased mortality and malformations as well as decelerated yolk-sac utilization (Eriksen et al., 2006; 2007). Here, assisted reproduction procedures may additionally influence steroid concentrations and their transfer to the offspring.

Thus, finding reliable markers to define egg quality at physiological, genomic, and proteomic levels continue to be a research aim to unravel the underlying mechanisms and processes attaining fertilization capacity and embryonic developmental competence.

1.3. Offspring performance and larval culture

Marine fish larval stages are characterized by a huge growth potential but also by high vulnerability. Thus, larval rearing and the commercial production of high quality juveniles still represents a major challenge for many aquatic species (Vadstein et al., 2018). Especially marine fish larvae are highly dependent on ideal biotic and abiotic conditions during their first stages of development in order to survive and grow properly (Hamre et al., 2018). Here, the microbial environment of fish larvae is defined by high bacterial load, which is mainly originating from organic material released to rearing systems (Vadstein et al., 2018). The interactions between host and microbes are still poorly understood but bacterial pathogens are known to cause disease outbreaks and thus high mortality (Vadstein et al., 2004). Fish embryos and larvae are free-living organisms that rely on their immune system to protect themselves against microorganisms. During embryonic and early larval development, fish solely depend on the innate arm of their immune system, comprising passive immunity through maternally transferred factors (Swain and Nayak, 2009; Uribe et al., 2011). In this phase, marine fish larvae are especially vulnerable to pathogens until the adaptive immune system is fully developed and functional (Uribe et al., 2011; Vadstein et al., 2018). Besides the biotic conditions, the biophysical conditions are of critical importance for early development (Howell and Baynes, 2004). Here, hatchery technology aims for optimized rearing conditions in terms of temperature, salinity, light, and oxygen that largely affect survival and growth (Yúfera, 2018).

Vulnerability of early larval stages further include the dependency on maternal factors in form of required nutrients that have been incorporated into the egg during vitellogenesis and are provided through the yolk sac (Izquierdo et al., 2001; Migaud et al., 2018). Larval survival, growth, and developmental abnormalities have been related to broodstock nutrition in various teleost species (Izquierdo et al., 2001). Studies on the composition, utilization, and larval retention of yolk nutrients may further help to understand the nutritional requirements of the larvae once exogenous feeding commences (Hamre et al., 2018). During early stages, marine fish larvae undergo major morphological and molecular changes including the development of the gastrointestinal tract (Zambonino Infante and Cahu, 2001). Here, the digestive potential appears to be genetically programmed through expression profiles of digestive enzyme, which may then be enhanced, stopped, or delayed depending on dietary composition (Zambonino Infante and Cahu, 2001). Providing the right nutrients according to dietary requirements of the larvae is crucial for larval ontogeny. Moreover, first feeding of larvae is highly dependent on chemical and physical characteristics of the offered diet in relation to taste, smell, size, motility, and buoyancy (Rønnestad et al., 2013). Increased knowledge on these critical transition phases during early life history is needed in order to optimize rearing methodologies to obtain viable and healthy larvae and juvenile fish. All phases from broodstock establishment and selection via reproductive protocols to larval culture need to be optimized to accomplish a closed-cycle production and domestication of any fish species in aquaculture.

2. European eel

While many cultured species fulfill the requirement of successful controlled reproduction for a sustainable aquaculture, industries of some important commercial species rely exclusively on wild-caught juveniles – among those is the European eel, *Anguilla anguilla*. The vast cultural and economic value in combination with a diminishing natural stock encourage initiatives to breed it in captivity. However, despite 100 years of research, knowledge on the eel life cycle remains incomplete with numerous open questions, in particular on the natural reproduction and early life history. For a sustainable aquaculture of eel, targeted, intensive research is needed especially towards areas related to broodstock nutrition, captive reproduction methods, as well as culture of embryos and larvae. Only through crosscutting research are we able to promote a captive production of eels.

2.1. Life cycle

The catadromous European eel poses a remarkable life cycle including various continental and oceanic life stages. The transition between these involves one of the longest migrations among fish, which until today remains partially disclosed (Figure 2). The spawning site of this species has been delimited to the Sargasso Sea (Schmidt, 1923). This assumption is based on the presence of early larval stages, as no spawning female or spawned eggs have ever been captured in the wild. Presumably, the eels do not leave the Sargasso Sea after spawning, but die after their reproduction (Tesch, 2003). Thus, knowledge on the natural reproductive development, spawning, as well as egg and embryonic stages is lacking, while information on early larval stages is scarce (Tesch, 2003). Known stages include the feeding larval stage, i.e. leptocephalus larvae that are being transported by currents such as the Gulf stream and North Atlantic Drift back to the continental shelves along Europe and

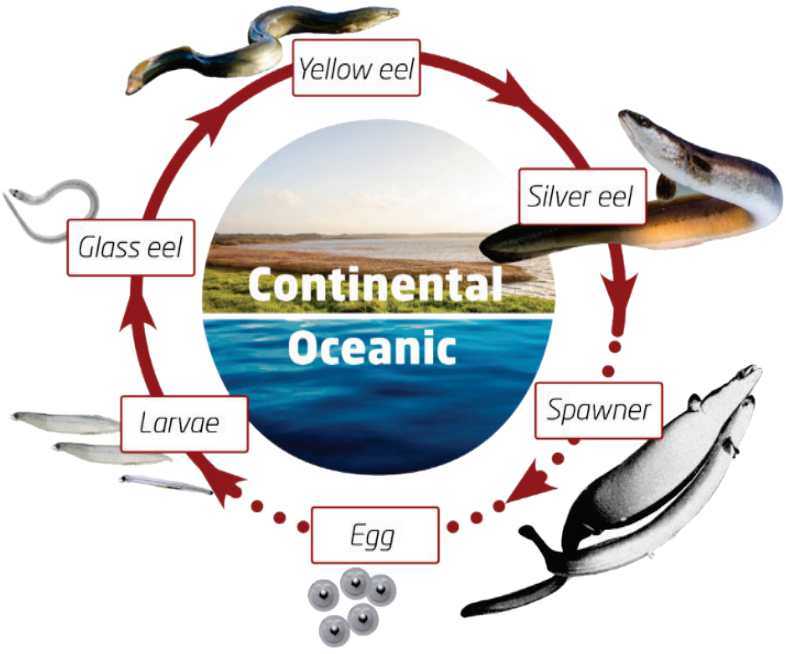
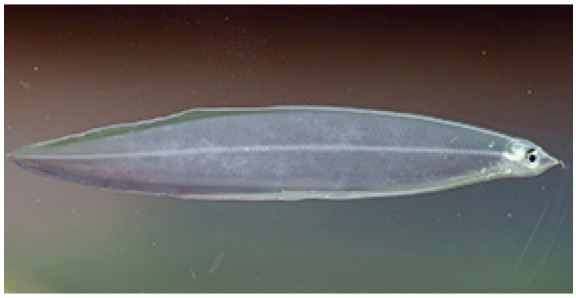


Figure 2. Life cycle of the European eel, *Anguilla anguilla* including life history stages related to oceanic and continental phases. The solid line represents the known part from nature, while the dashed line indicates still unknown parts addressed in experimental research. Graphic: Adapted from Tomkiewicz et al., 2019.

A



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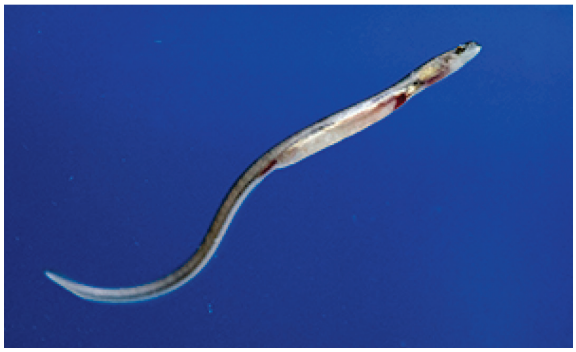

Figure 3. Offspring of European eel, *Anguilla anguilla*, (A) wild-caught leptocephalus larvae showing the peculiar leaf-like body shape characteristic for elopomorph species and (B) the elver stage in which the glass eels have gained pigmentation and found foraging habitats. Photos: (A) Sune Riis Sørensen (B) Tao Lytzen.

North Africa at a size of 45-75 mm (Munk et al., 2010). The larval drift is expected to last for about 200-300 days by the end of which the larvae metamorphose into their transparent juvenile stage, called “glass eels” (Figure 3). Whilst inhabiting freshwater and coastal habitats, the glass eels develop into the fully pigmented elver stage and subsequently into yellow eels, named after their yellow-greenish color. The growth period in freshwater and coastal habitats lasts around 2-15 years for males and 4-20 years for females. Following this growth period with an increase in body fat, eels transform into silver eels, through a process called “silvering” that is associated with the onset of spawning migration.

The silvering process includes a series of morphological and physiological changes including darkening of the dorsal part to a bluish or brownish black, while the belly turns silvery white. Further changes include elongated pectoral fins, enlarged eyes, and thickened skin (Durif et al., 2005). These modifications prepare eels for the oceanic life, as e.g. the enlarged eyes allow more efficient absorption of light, while the changes in skin color ensure decreased visibility to predators (Durif et al., 2009). Concurrently, a dopaminergic inhibition prevents both sexes from undergoing gametogenesis whilst inhabiting coastal habitats. During silvering, eels enter a prepubertal stage associated with initial gamete development, slight increases in the gonadosomatic index (GSI), sex steroid concentrations, as well as vitellogenin plasma levels (Dufour et al., 2005, 2003). Moreover, the digestive tract regresses, as eels cease feeding concurrent with initiation of their oceanic migration (Tesch, 2003). In addition to the morphological changes, silvering also includes changes such as an increase in the gonadosomatic index and concentrations of sex steroids, which are related to the onset of maturation (Dufour et al., 2003; Rousseau et al., 2009). Eels show high variability in age and size at silvering indicating flexible maturation strategies. Nonetheless, if the nursery area and natural conditions allow, then eels show a tendency to attain larger body sizes before onset of migration (Yokouchi et al., 2018).

2.2. Stock abundance and exploitation

Once abundant and ubiquitous, European eel is now considered a critically endangered species (Jacoby and Gollock, 2014). Widespread fisheries, targeting all continental life stages have reduced the population. Furthermore, the life cycle of European eel involves several habitats, all of which are greatly impacted by human activity. Hence, eels are affected by migration barriers (e.g. hydropower turbines), pollution, diseases introduced by humans, and climate change (Bonhommeau et al., 2008; Friedland et al., 2007; Gutiérrez-Estrada and Pulido-Calvo, 2015; Knights, 2003; van den Thillart et al., 2009). As a consequence, recruitment of glass eels and hence abundance of yellow eels have strongly declined since the 1960s (Figure 4). Thus, a drastic decline is found for glass eel recruitment in European waters, which in 2018 was only ~2 % of the mean level from 1960-1979 in the “North Sea” series and ~10 % in the “Elsewhere Europe” series (Figure 4A). Overall, the recruitment of yellow eels in 2018 was reduced to 29% of the mean level from 1960-1979 (Figure 4B).

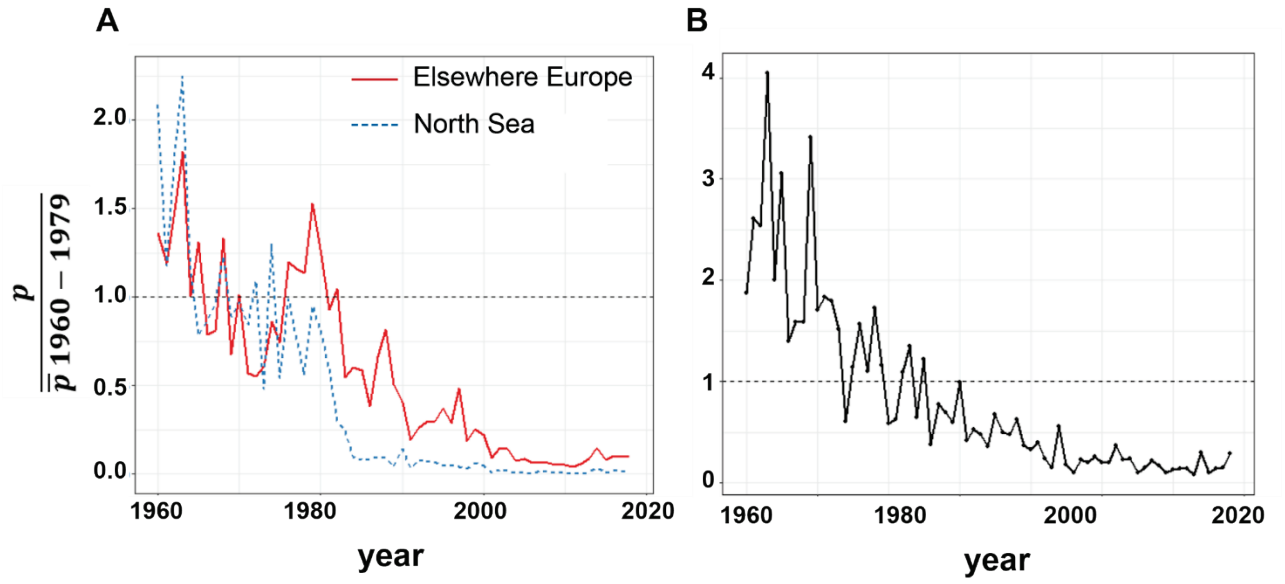

Figure 4. (A) Geometric mean of estimated (GLM) (A) glass eel recruitment for the continental North Sea and Elsewhere Europe series and (B) yellow eel recruitment in Europe updated in 2018. The GLM (glass eel ~area: year + site) predictions were scaled to the 1960-1979 average. Adapted from ICES, 2018.

Exploitation of eels dates far back in time. Thus, fisheries has a long history and tradition in European countries, evolving from being poor man’s food, locally captured, to a species considered a delicacy with high market price. As a species of high economic value, eel aquaculture started growing towards the end of the 1980s to 1990s. This was a result of the development of resource efficient RAS technology for outgrowing to commercial size, in combination with eel being a social species performing well at high densities. The aquaculture production of European eel in Europe increased until mid-2000s to levels of 8000-9000 t. Thereafter, while aquaculture of other species has developed, the production

of European eel decreased to 5000-6000 t (Figure 5). This decline relates to that aquaculture is capture-based and relies on wild-caught glass eels. Decreasing availability and increasing prices urged the eel aquaculture to target the development of reproduction methods and larval culture technology for glass eel hatcheries. Thus, establishing hatchery practice would enable a closed-cycle production and domestication of eel, a goal that has been a target over more than two decades for both, Japanese, *Anguilla japonica* and European eel.

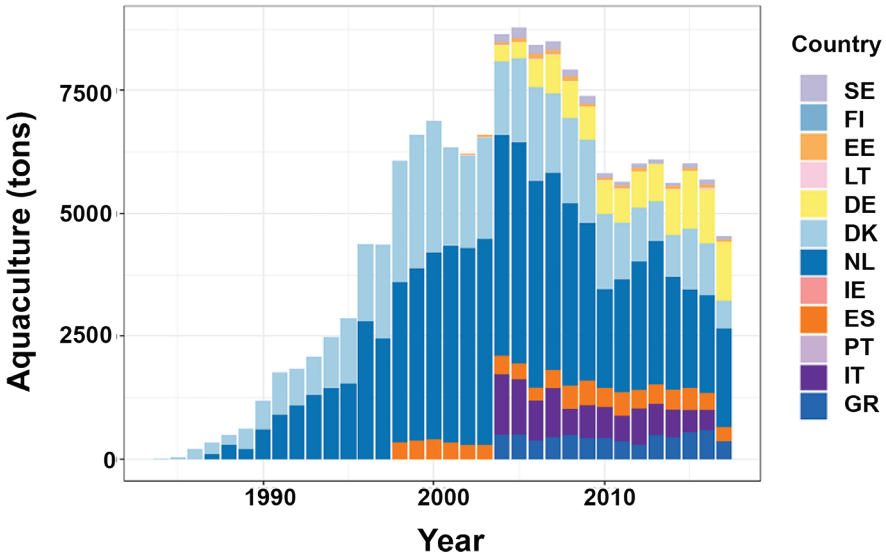

Figure 5. Reported aquaculture production of European eel in Europe from 1984 onwards, in tonnes, in Sweden (SE), Finland (FI), Estonia (ES), Lithuania (LT), Germany (DE), Denmark (DK), Netherlands (NL), Ireland (IE), Spain (ES), Portugal (PT), Italy (IT) and Greece (GR). Adapted from ICES, 2018.

2.3. Hatchery development

Natural triggers for maturation of European eel is scarcely known and a strong inhibitory hormonal control prevents the European eel from undergoing sexual maturation in their foraging habitats of European continental waters and so in captivity (Vidal et al., 2004). Consequently, hatchery technology of eels needs to develop and adopt assisted reproduction treatments including the administration of gonadotropins to induce gamete development as well as steroids to induce the follicular maturation. Experimental research on captive breeding of European eel has a long history, driven by the curiosity about the complex reproduction biology. The first publication of successfully induced maturation in male eels originates from a French group in the 1930s (Boucher, 1934; Fontaine, 1936), with later development of the protocols for female eels (Boëtius and Boëtius, 1967; Fontaine, 1964). Still, production of viable offspring was difficult and not until 35 years ago,

the first larvae obtained from European eel using hormonal treatments were reported by Russian scientists (Bezdenezhnykh, 1983). However, following this breakthrough, research stunted for the next 20 years. It was not until a Danish researcher applied protocols developed for the reproduction of the Japanese eel that a successful production of European eel larvae was achieved (Pedersen, 2003; 2004). This achievement in combination with the first successful production of leptocephali and later glass eel for Japanese eel revived the research interest developing hatchery technology for a future aquaculture (Tanaka et al., 2001; 2003). Henceforth, increasing studies on the successful reproduction and developing standardized protocols were acquired for the European eel (Mordenti et al., 2013; Palstra et al., 2005; Sørensen et al., 2013; Tomkiewicz, 2012; Tomkiewicz et al., 2019).

Figure 6. EEL-HATCH facility located in Hirtshals, Denmark. Photo: Sune Riis Sørensen.

Within the framework of the Danish innovation project EEL-HATCH "*Eel Hatchery Technology for a Sustainable Aquaculture"*, breeding technology was brought to the next step by establishing a state-of-the-art-prototype-hatchery for European eel in Hirtshals, Northern Jutland, Denmark, which was built within the project (Figure 6). Here, promising new achievements towards closing the life cycle have been accomplished including first experiments to establish feeding culture of European eel larvae (Butts et al., 2016; Politis et al., 2018c; Tomkiewicz et al., 2019). Since 2018, the on-going project ITS-EEL "Improve technology and scale-up production of offspring for European eel aquaculture" is advancing technologies and techniques aiming at the hatchery production of glass eels to enable a closed-cycle production of European eel for the aquaculture industry.

2.4. Broodstock nutrition

Studies on dietary impacts on eel broodstock and their reproductive success are limited to Japanese eel female broodstock (Furuita et al., 2006; 2007), European eel female broodstock (Støttrup et al., 2013; 2016), and European eel male broodstock (Baeza et al., 2015a; 2015b; Butts et al., 2015; 2019). European eel cease feeding during their long

migration to spawning grounds in the Sargasso Sea. Thus, the integration of dietary components impacting reproductive success and offspring quality needs to take place prior to induction of sexual maturation and gamete development. For female eel, the accumulation of lipids in form of oil droplets in oocytes (lipidation) is initiated already in primary oocytes during the immature stage. For completing the process together with vitellogenesis induced by hormonal treatments, nutrients accumulated in muscle, viscera, etc. are utilized. Information on the nutrient composition of spawning females, eggs, embryos, and yolk-sac larvae from the spawning area is not available. Hence, analyses of eggs obtained from wild-caught silver eels through assisted reproduction trials need to serve as baseline to alter nutritional composition of diets for farm-raised broodstock. Here, differences in lipid content and fatty acid profiles were found between farm-raised and wild-caught females, in particular in levels of ARA, EPA, and DHA (Støttrup et al., 2013). For instance, higher ARA and lower EPA levels have been found in wild-caught compared to farm-raised eels (Heinsbroek et al., 2013; Støttrup et al., 2013). However, altering EFA levels in farm-raised diets has successfully enhanced the composition in ovary, visceral fat or muscle tissue of female European eel. Here, a long feeding duration was required to alter the EFA composition with gradual increases over 44 weeks of feeding period (Støttrup et al., 2013). A second study revealed that elevated ARA levels in muscle and ovarian tissue due to enhanced diets resulted in enhanced production of embryos and larvae (Støttrup et al., 2016). However, this study was quantitatively measured on a binomial scale and did not provide detailed insights on the effect of broodstock nutrition on the offspring quality. Moreover, n-3 LC-PUFA enhancement in the diets appeared to promote oocyte growth during induced vitellogenesis (da Silva et al., 2016). In Japanese eel, ARA levels between 2.8 and 4.0 % of total FA in the unfertilized eggs represented high quality, whereas too high ARA levels (4.6 – 5.6 % of total FA) had a negative effect on offspring performance (Furuita et al., 2006; 2007). Also, an overall too high ratio of n-6 to n-3 fatty acids negatively impacted embryogenesis (Furuita et al., 2007). Moreover, optimizing levels of Vitamin A, C and E in combination with highly unsaturated fatty acids (HUFA) led to enhanced egg and larval quality (Furuita et al., 2009a; 2009b). On the contrary, in European eel, increased dietary Vitamin C and E levels did not impact levels of lipid peroxidation products in the eggs, indicating that even lowest tested dietary vitamin levels were sufficient (Støttrup et al., 2016).

Also in male European eel, LC-PUFA are expected to play an important role during induced sexual maturation (Mazzeo et al., 2010). Here, the importance of ARA, EPA, and DHA in male broodstock diets was shown on volume of milt and sperm motility (Baeza et al., 2015a; Butts et al., 2015). The importance of fatty acids and their dynamics throughout spermatogenesis further revealed the importance of the effect of lipid metabolism (Baeza et al., 2015a; 2015b). Furthermore, certain amino acids have been shown to impact sperm performance traits in European eel (Butts et al., 2019).

2.5. Assisted reproduction

The availability of high quality broodstock and gametes is crucial for the establishment of breeding programs with the aim to find parental combinations with the best possible traits to enhance offspring survival, growth, and disease resistance (Gjedrem, 2010). Maternal effects are often considered to dominate paternal effects (Chambers and Leggett, 1996). In European eel, the importance of compatibility between female and male broodstock on early offspring performance has been described (Benini et al., 2018). Nonetheless, the study also confirmed that maternity is the major factor influencing these early life history traits.

As mentioned before, the continental phase of the European eel life cycle is characterized by the dopaminergic inhibition of gonadotropic function, to be exact the production and release of FSH and LH (Vidal et al., 2004). The mechanisms behind this endocrine control have been studied extensively since the beginning of this century and in particular the inclusion of molecular tools has delivered new insights into the complex function (e.g. Ager-Wick et al., 2013; Campo et al., 2018; Lafont et al., 2016; Morini et al., 2015; Pasquier et al., 2012; 2018; Rojo-Bartolomé et al., 2017). Despite advanced research, current techniques to induce sexual maturation and vitellogenesis of female European eel in captivity includes the administration of exogenous gonadotropins of either carp or salmon pituitary extracts (PE; Figure 7). The first viable offspring of European eel was obtained by applying biweekly injection of carp pituitary extracts (CPE) (Bezdenezhnykh, 1983). Subsequently, most applied experimental research has used weekly injections, whereas the dosage and type of gonadotropin varied between salmon pituitary extracts (SPE) (da Silva et al., 2016; Pedersen, 2003; Tomkiewicz, 2012) and CPE (Mordenti et al., 2013; Palstra et al., 2005; Pérez et al., 2011). Commonly, a dosage of around 20 mg PE kg^{-1} initial body weight (BW)

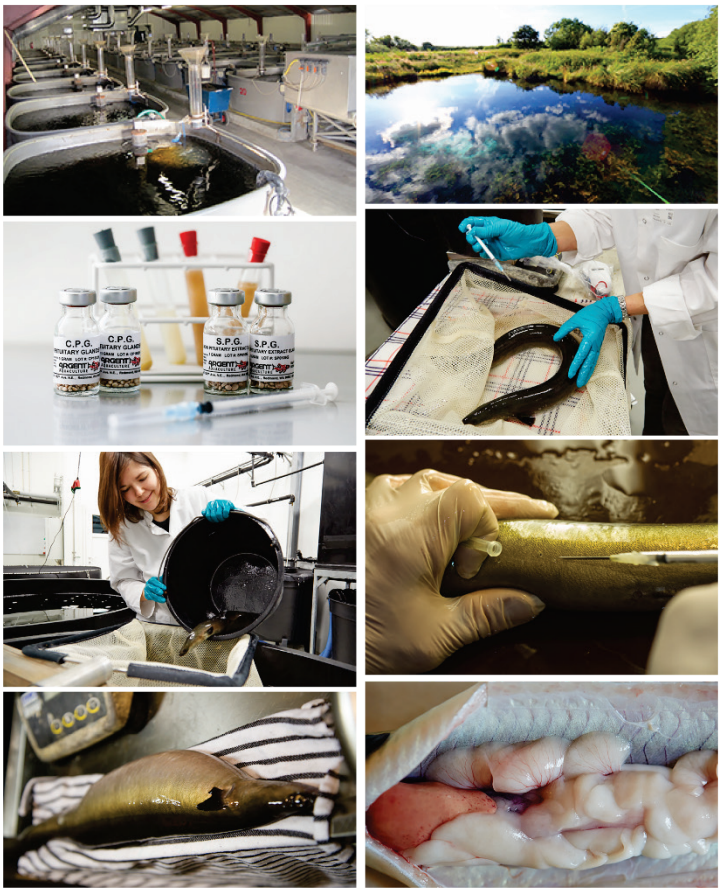

Figure 7. Broodstock assisted reproduction methodologies. Upper panel left: Christian Graver; Upper panel right: Henrik Egede Lassen; Following: Sune Riis Sørensen.

is used to initiate the ovarian development between 12-20 weeks. However, CPE is also used in increasing doses throughout development (Di Biase et al., 2016; Mordenti et al., 2012). Changes in dosages have been tested for SPE but only affected progression of ovarian development in combination with enhanced LC-PUFA levels (da Silva et al., 2016). Moreover, environmental conditions, such as light, temperature, and swimming can modulate the ovarian development (Mazzeo et al., 2014; Mordenti et al., 2012; Palstra and van den Thillart, 2009; Pérez et al., 2011). Additionally, co-treatment with androgens during artificial maturation of female European eel has shown potential to improve reproductive success and offspring quality (Di Biase et al., 2017).

During oogenesis, steroids play a major role in teleost, which has also been shown for anguillid species (Burgerhout et al., 2016; da Silva et al., 2016; Kazeto et al., 2011). For instance, in the Japanese eel, the importance of E2 and 11-kt for previtellogenic and vitellogenic growth of oocytes was shown, while DHP plays a major role in the induction of follicular maturation (Ijiri et al., 1995; Kazeto et al., 2011; Matsubara et al., 2005). Likewise, in European eel, the importance of sex steroids and their dynamics has been observed with increasing levels of E2, T, and 11-kt throughout sexual maturation (Burgerhout et al., 2016; da Silva et al., 2016). After reaching oocyte growth by administration of PE, successful follicular maturation and ovulation require the use of a MIS, often preceded by a priming dose of PE (Nagahama and Yamashita, 2008). Here, the most commonly used steroid is DHP. The timing of MIS provision is of high importance and indicators include female body weight, oocyte appearance, staging development according to germinal vesicle migration, and coalescence of oil droplets (da Silva et al., 2018; Kagawa et al., 2017; Palstra et al., 2005). Wrong timing in the induction of spawning may lead to non-successful ovulation, low quality, i.e. non-floating eggs, or low fertilization capacity. Among several challenges during the assisted reproduction treatment is the asynchronous or group synchronous oocyte development, which indicates that eels are batch spawners. Batch fecundity is high in the European eel, ranging from 0.7 to 2.6 million eggs per female while being similar between 1st and 2nd batch (Boëtius and Boëtius, 1980; Tomkiewicz, 2012). This high egg production provides great potential for possible future aquaculture purposes.

For male eel, spermatogenesis is commonly induced by weekly injections of human chorionic gonadotropin (hCG) with spermiation starting as early as week five (Asturiano et al., 2006; Pérez et al., 2000). While spermatogenesis may already be induced by a single-dose injection, highest efficiency and sperm quality may be obtained through weekly injections and a priming dose administered 24 h before stripping (Asturiano et al., 2005; Ohta et al., 1997). Also for male eels, temperature has been shown to affect the progression of spermiation (Baeza et al., 2014; Peñaranda et al., 2016). Methods of long-term storage of eel sperm have been investigated leading to the development of cryopreservation protocols (Herranz-Jusdado et al., 2019a). Moreover, research has focused on developing recombinant gonadotropins to induce spermatogenesis and evaluating this alternative to application of PE as products of animal origin. Here,

spermatogenesis and spermiation may be induced by use of eel specific recombinant gonadotropins and hold great future potential. However, treatments still need to be adjusted to reach the same sperm quality as obtained through standard protocols (Herranz-Jusdado et al., 2019b; Peñaranda et al., 2018).

2.6. Egg quality and early life history

In nature, females release the eggs into the aquatic environment, where they are being externally fertilized and activated under hyper-osmotic conditions (Browne et al., 2015). In the eel hatchery, both spontaneous spawning and manual stripping are used to obtain eggs (Di Biase et al., 2016). However, in this project, we utilize manual stripping of the eggs and in vitro fertilization with pre-stripped milt kept in an immobilizing medium (Butts et al., 2014; Sørensen et al., 2013). This allows to establish fertilization protocols, where the sperm-to-egg ratio is calculated, securing standardized conditions for every female in order to reduce noise in experimental tests. Eggs are subsequently incubated in artificial or natural treated seawater (Sørensen et al., 2015). Eel is a pelagophil species, producing buoyant fertilized eggs that contain multiple lipid droplets gradually fusing together to one large lipid droplet. The embryonic development of European eel is illustrated in Figure 8. During the first hours after fertilization, the chorion separates from the plasma membrane, forming the perivitelline space (PVS) (Sørensen et al., 2016). This swelling of the egg is important for later development of the

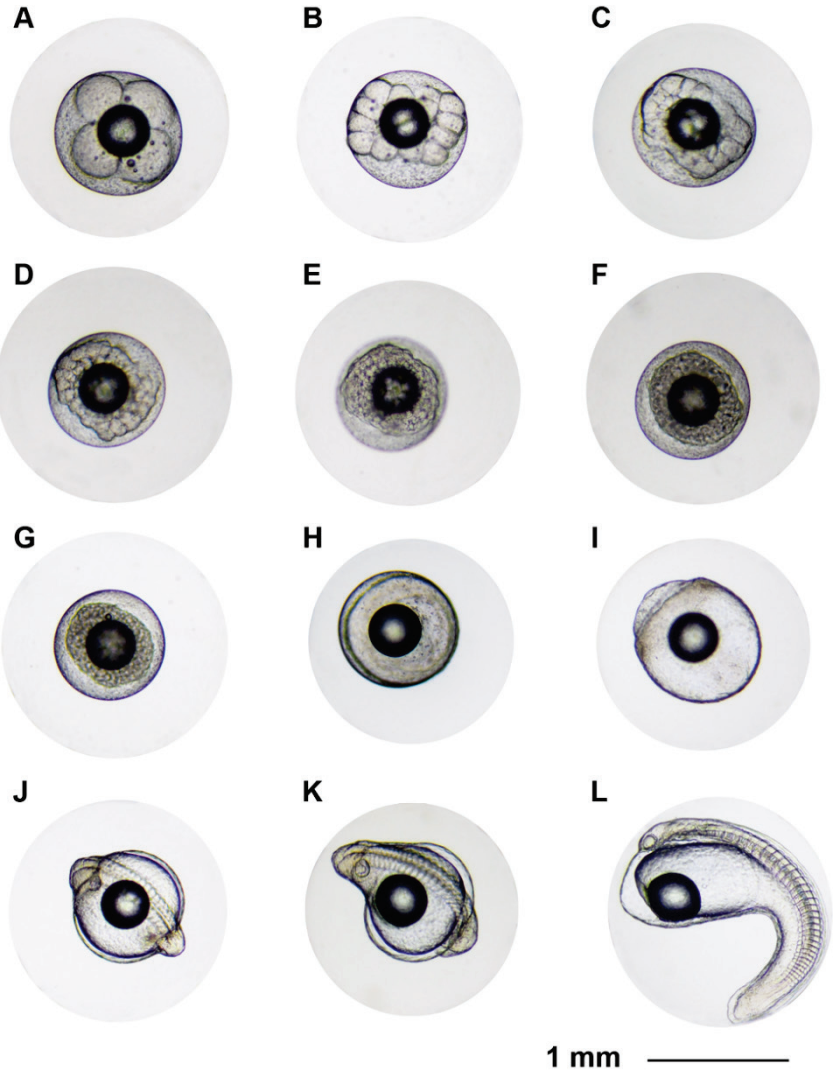

Figure 8. Stages of embryonic development in European eel, *Anguilla anguilla* at 18 °C. (A) 2 hours post fertilization (hpf), (B) 3 hpf, (C), 4 hpf, (D), 5 hpf, (E) 6 hpf, (F) 7 hpf, (G) 8 hpf, (H) 16 hpf, (I) 24 hpf, (J) 32 hpf, (K) 40 hpf, (L) 48 hpf. Scale bar 1 mm.

embryo including the process of hatching. The first cell cleavages become visible at ~1 hour post fertilization (hpf) and within ~4 hpf, the 16 cell stage is reached. The early blastula stage is reached ~8 hpf and from here on, the embryonic disc is formed, making it impossible to distinguish between the cells. This is followed by the formation of the germ ring at ~14 hpf characterizing the onset of gastrulation. After ~15 hpf, the gastrula comprised 50 % of the yolk, defined as ½ epiboly. The onset of segmentation with first visible somites commences at ~24 hpf, while at 32 hpf, several somites are visible and the embryo develops two distinct eye capsules. Hereafter, the tail bud evolves and the yolk sac becomes ellipsoid. During the last hours prior to hatch, the embryos move frequently until hatching occurs at ~56 hpf at 18 °C (Sørensen et al., 2016). Natural conditions throughout this developmental stage are undiscovered in nature and ideal rearing conditions consequently need to be identified through experimental studies in a hatchery like EEL-HATCH. Recent achievements have shown an optimal temperature of 18 °C and a salinity of 36 PSU (Politis et al., 2017; 2018a) during embryonic development. Moreover, a 12h-12h dark/light photoperiod under low light intensity leads to highest embryonic survival and hatch success (Politis et al., 2014). To a large extent, the embryonic development is influenced by maternal factors such as nutrition in form of yolk including the oil droplet and cytoplasmatic factors, such as maternal mRNA and hormones transferred by the motherfish (Lubzens et al., 2017). A critical bottleneck throughout embryonic development may be the MZT, which takes place at ~10 hpf in European eel at 18 °C (Sørensen et al., 2016). A first study on European eel has looked at the mRNA abundance throughout embryonic development comparing hatch and no-hatch groups (Rozenfeld et al., 2016). Here, the mRNA abundance between groups did not differ at 0 and 5 hpf, however, at 30 hpf the hatch group showed higher abundance of five genes analyzed (*cpt1a*, *cpt1b*, *β-tubulin*, *phb2*, and *pigf5*). This indicates the importance of the embryo's own upregulation of these genes after the mid-blastula transition.

Larval development throughout the yolk sac stage is illustrated in Figure 9. European eel larvae hatch as relatively undeveloped larvae with distinct yolk sac and large oil droplet, contributing to attaining neutral buoyancy. Subsequently, larvae start utilizing the oil droplet to grow in length. Moreover, morphological changes include visible optic capsules and hindbrain, broader primordial fins, and a well-defined and pigmented tail. At 8 days post hatch (dph), the upper and lower yaw formation starts and the eyes become pigmented. Finally, the forming of the feeding apparatus of first-feeding larvae with characteristic, protruding teeth starts and the yolk sac is fully consumed (Sørensen et al., 2016).

Figure 9. Early larval development of European eel, *Anguilla anguilla* at 18 °C. (A) larva at hatch, (B) 2 days post hatch (dph), (C) 4 dph, (D) 6 dph, (E) 8 dph, (F) 10 dph, (G) 12 dph, (H) 14 dph. Adapted from Politis et al. 2017.

The natural environmental regime of yolk sac larvae of European eel remains unknown and experimental studies have investigated optimum rearing conditions, in terms of microbial activity, light, temperature, and salinity. Here, Sørensen et al. (2014) studied the effect of microbial control and disinfection treatments on eggs revealing that low microbial activity resulted in higher embryonic survival, hatch success, as well as larval survival. Thus, optimal incubation of European eel is based on a low-level, stable bacteria community during this sensitive life stage. Furthermore, eel larvae have been shown to be sensitive to all physical parameters of light (Politis et al., 2014). Here, larvae reared under low intensity light showed higher survival that those under high intensity light, larvae reared under 12h-12h dark/light regime showed higher survival than larvae reared under constant light, and larvae reared under red light showed higher survival than those under white or green light. Furthermore, Politis et al. (2017) identified the optimal rearing

temperature for European eel larvae at 18-20°C with efficient growth and low abundance of deformities. Moreover, expression of growth hormones was high, indicating better growth and expression of heat shock proteins was low, indicating low stress levels. Finally, the impact of different salinity reduction regimes towards iso-osmotic levels was tested (Politis et al., 2018a). Here, a gradual decrease in salinity from 36 to 18 PSU resulted in increased survival and growth and decreased appearance of severe deformities.

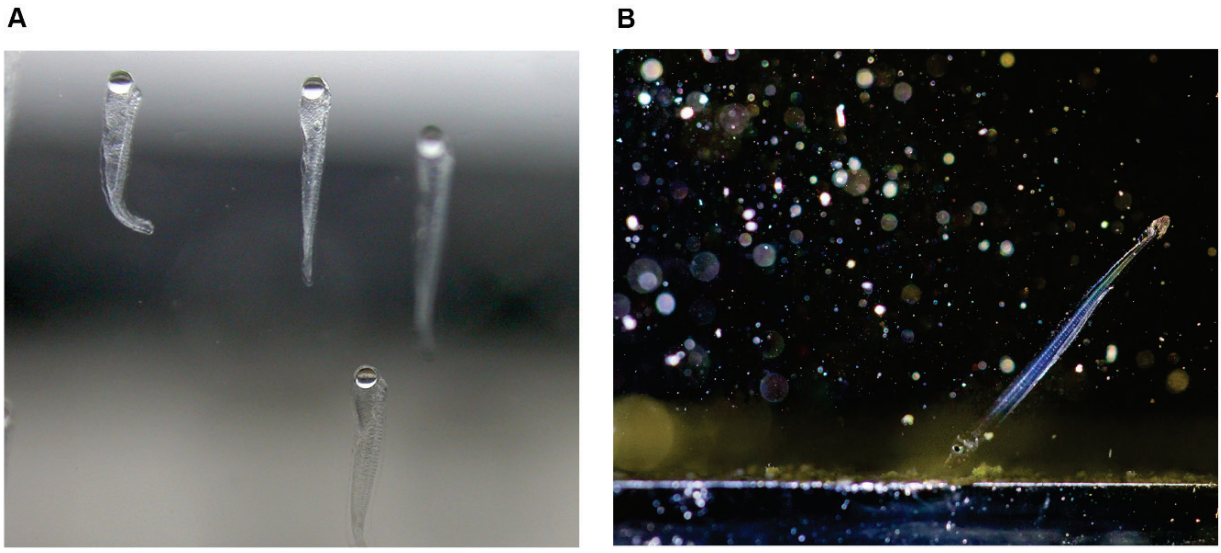

Figure 10. European eel, *Anguilla anguilla* larvae. (A) Newly hatched larvae, (B) feeding larvae. Photos: Sune Riis Sørensen.

For many years, first-feeding of larvae was one of the main bottlenecks of European eel research and knowledge on the natural feeding requirements was lacking. Within the last ten years, advanced analyses of stomach content of larvae caught in the Sargasso Sea has given insights into some dietary sources (Ayala et al., 2018; Riemann et al., 2010). Nonetheless, knowledge on nutritional requirements of the larvae is scarce and extensive feeding trials need to be conducted. These efforts have led to the first feeding experiments for European eel larvae (Butts et al., 2016; Politis et al., 2018c; Figure 10). Future research will focus on optimizing larval diets to establish growth and feeding larval culture of European eel.

3. Gaps in knowledge and aim of the studies

In recent years, a stable production of viable eggs and larvae of European eel has been attained at the EEL-HATCH facility. Still, egg quality is variable, in particular for farm-raised broodstock, affecting the proportion of females successfully spawning and the quality of the eggs produced, considering viability of eggs and larvae. However, a predictable production of high quality eggs from farm-raised female broodstock is required for closing the life cycle in captivity and establishing an efficient hatchery production. In this context, there are three main aspects influencing gamete production and quality. The first concerns female nutrition, where stored reserves need to be transferred to the egg and embryo. The second and third study relate to assisted reproduction procedures, which include on the one hand, induced vitellogenesis for the development of yolked oocytes, and on the other hand, follicular maturation where the oocyte resumes meiosis, gain fertilization competence, and finally the egg is ovulated. Along the progress of hatchery technology development, studies on influences of nutrition and assisted reproduction on egg quality and offspring developmental competence have entered a new era allowing quantitative investigations and analyses for the European eel. Here, the experimental studies included in this PhD project aimed at substantiating knowledge on the nutritional and reproductive physiology in association with assisted reproduction and the impact of maternal factors on egg quality and offspring performance in European eel.

Broodstock nutrition is known to influence reproductive performance and offspring quality through maternal influences on egg biochemical composition. Here, Study 1 focused on enhancement of broodstock diets for improved egg quality from farm-raised broodstock, applying wild-caught female broodstock as benchmark. Based on previous findings, the objective was to elucidate offspring requirements of EFA (ARA, EPA, DHA) through manipulation of maternal diet composition and quantification of fatty acid composition of eggs and larvae. Furthermore, new insight into offspring utilization of EFA and impact on fatty acid levels and ratios on offspring developmental competence and survival was targeted. Overall, three diets were designed and the impact on offspring performance was studied. Thus, eggs and larvae were sampled and multiple parameters assessing quality and developmental success were analyzed to identify critical stages. Moreover, the effect of maternal feeding period on egg EFA composition was investigated comparing size-matched females from two feeding regimes. In aquaculture, growth rates are generally highly variable and fast growth is usually targeted. However, due to trade-offs in allocation of resources to growth and reproduction (Folkvord et al., 2014), fast growth may not necessarily favor reproductive success and offspring quality. As eels are not fed during their reproductive development, it is particularly important to substantiate insights into required EFA levels and ratios to optimize future diet formulation for farm-raised female broodstock as well as finding ideal feeding regimes. Complementing previous findings, the aim was to acquire new understanding for future improvements in farm-raised broodstock diets and management required for future closed-cycle production.

Currently, hormonal treatments by application of exogenous gonadotropins are required in order to initiate sexual maturation and sustain gamete development in breeding protocols of European eel. While effects are species-specific, such hormonal applications, although serving the purpose, may have unintended impacts on egg and offspring quality (Mylonas et al., 2010). Hormonal treatment protocols for the induction of vitellogenesis in female European eel vary within and between research groups but common to present therapies, is that they apply CPE or SPE. However, the composition of applied pituitary extracts, inclusive contents of active gonadotropins, FSH and LH, and their potential effect on egg and offspring quality is unknown. Here, the objective of Study 2 of this PhD project was to gain insights into molecular processes related to experimental induction of vitellogenesis using two hormonal treatment schemes and evaluating their effects on female maturation, egg quality, and embryonic development. As such, this study compared the effect of applying exogenous gonadotropins, either CPE or SPE with sampling of ovarian tissue, unfertilized eggs, and embryos at few hour's intervals until hatch. The focus of molecular analyses was on mRNA transcript abundance of genes important for embryogenesis with particular emphasis on the MZT which is little studied in eels and may be impacted by the induction procedures.

While repeated administration of exogenous gonadotropins induces ovarian development, a further critical step is the induction of follicular maturation, which is also impeded by the complex endocrine mechanisms in eel. As such, follicular maturation and the acquisition of fertilization competence of the oocyte is controlled by MIS. Here, assisted maturation protocols initiating this process commonly apply an additional injection of PE (primer) to increase LH levels followed by administration of MIS. In this context, Study 3 aimed at exploring possible maternal transfer of sex steroids, their dynamics throughout embryogenesis and potential effects on the oocyte's ability to resume meiosis, form a zygote and develop into an embryo. In the embryo, hypothalamus–pituitary–interrenal (HPI) axis and *de novo* steroid synthesis is likely not initiated until after hatch, and early stages are defined by maternally transferred hormones (Nesan and Vijayan, 2013). However, excess hormones of maternal origin that are not metabolized by the embryo may have detrimental effects on their development. Thus, analyses of steroid concentrations on embryonic developmental competence in European eel were performed including E2, T, 11-kt, DHP, and cortisol, measured via radioimmunoassay. To gain further insights into the physiological processes, molecular analyses were applied aiming at defining the expression profiles of growth and development related genes involved in stress/repair, somatotropic axis, as well as lipid and thyroid metabolism. Several of these genes are important for early larval development of European eel (Politis et al., 2017; 2018a; 2018b; 2018c). However, knowledge is lacking on the maternal transfer and expression profiles throughout embryogenesis. Thus, this study provided insight into the maternal transfer and dynamics of steroids and gene expression throughout embryonic development as well as potential impact on their developmental competence that remains unexplored but deserves attention not least in viable offspring production context.

This PhD project combined fatty acid analysis, radioimmunoassay, and molecular tools to extend our knowledge on maternal factors impacting egg quality and offspring developmental competences with the aim to decode underlying processes. We targeted essential fatty acids, maternal transfer of mRNA transcripts, steroid dynamics, and gene expression throughout embryogenesis to increase our understanding. Together, these crosscutting analyses of experimentally obtained samples and data allowed detailed insight into underlying mechanisms of important developmental processes that complement morphological findings and defined parameters important for evaluation of egg and offspring quality in European eel.

4. Findings of my PhD

Overall, the studies of this PhD project have provided novel insight into maternal effects on egg quality and offspring developmental competence. These new insights comprise nutritional demands and hormonal treatments of female broodstock to optimize production of viable eggs and larvae. In the first study, reproductive success and offspring quality were experimentally compared for the first time between farm-raised and wild-caught females, providing valuable benchmarking information. At the same time it is the first study on eel to quantify impacts of EFA and feeding regimes on egg and larval composition, development and survival rates. Moreover, thorough observations throughout embryonic development helped to broaden our understanding of processes defining the most critical periods during early development. Here, declines in survival occurred during the MZT, where the developmental control is taken over by activation of the zygotic transcription. The successful process of this transition that is essential for embryonic development revealed a bottleneck in early life history of European eel that related to all three factors studied. Another limiting step in the development, was the occurrence of cleavage abnormalities during early embryonic development that highly impacted embryonic developmental competence and differed between broodstock origin and hormonal treatments used to induce sexual maturation and vitellogenesis. Complementing morphological findings, we used molecular analyses in the form of gene expression analysis, providing a measure of mRNA transcript abundance, which has become state-of-the-art methodology in studies of mechanisms underlying complex processes in developmental biology. These studies profiled the expression of important developmental genes from the ovary of the female, the unfertilized egg, and throughout the embryonic development until shortly before hatch, which generated new insights into the importance of maternal mRNA transcripts and their clearance in relation to MZT as well as the activation and expression of embryos own genome. We showed that the mRNA transcript abundance of maternal effect genes affected developmental competence in a way similar to other marine species. Here, expression of important genes was related to quality parameters, e.g. embryonic survival, cleavage abnormalities, or hatch success revealing their importance for early development in eel. Moreover, we found that sex steroids were

transferred from the female to the unfertilized egg and their concentrations and dynamics during embryonic development were related to developmental competence. Together, results of these studies point at an array of causes of varying egg quality and high embryonic mortality that can be implemented in various parameters to enhance reproductive success of European eel.

Study 1: 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

This study generated new knowledge on the impact of fatty acid composition of female broodstock diets and feeding regimes on egg quality and offspring developmental competence.

In teleosts, broodstock nutrition is known to play a key role for reproductive success and egg quality, as nutrients required for offspring development are incorporated into the egg prior to or during vitellogenesis (Izquierdo et al., 2001; Migaud et al., 2018). Fatty acids, in particular DHA, EPA, and ARA are essential in the case of marine fishes, and dietary supplies need to meet the embryos demands (Glencross, 2009; Sargent et al., 1995; Tocher, 2010). Previous published results indicate similar demands for European eel and thus, a long feeding duration is required, as eels in nature cease feeding at onset of their long spawning migration to the Sargasso Sea (Støttrup et al., 2013; 2016). As eels are accordingly not fed during induced vitellogenesis, dietary supplementation of fatty acids in adequate amounts and proportions needs to be provided for accumulation in muscle, viscera, and ovary during the immature stage.

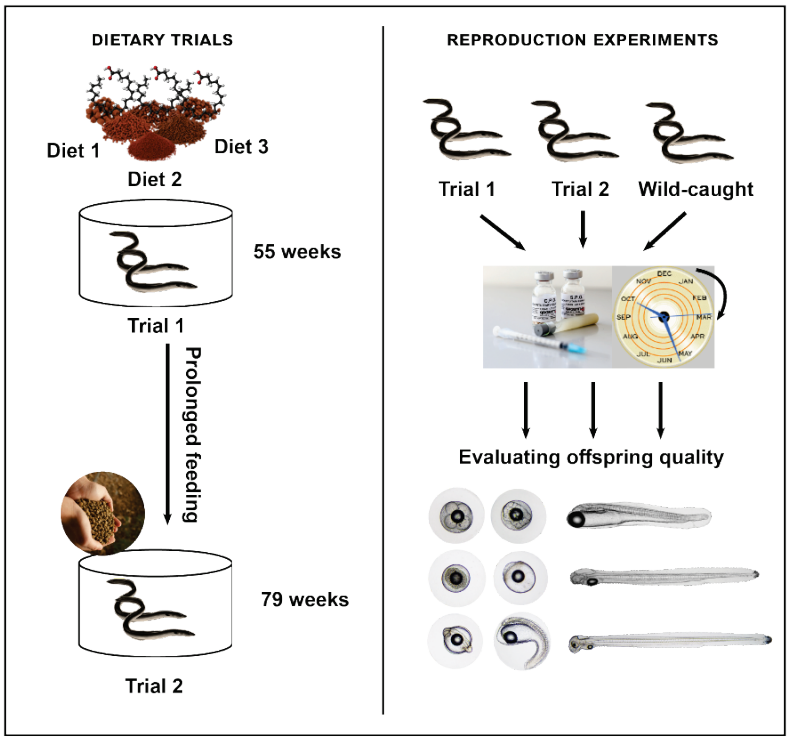

Figure 11. Simplified schematic of the experimental set-up of dietary trials and reproduction experiment for Study 1.

This study included three reproduction experiments using size-matched females, two of these comprised farm-raised broodstock fed different diets for different periods of time and one wild-caught broodstock.

The formulated diets varied in levels and ratios of three EFA, 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. The maternal dietary effects of EFA on egg fatty acid composition, egg quality, and offspring development were evaluated. At last, results on offspring quality of the best performing diet were compared to those of wild-caught females to benchmark results. A simplified schematic overview of the experimental set-up of the study is shown in Figure 11.

Highlights of the study:

- Diets enhanced in DHA and ARA composition increased the total lipid content of eggs, the amount of floating eggs, fertilization success, and embryonic survival;
- Longer feeding duration and further EFA enhancement led to higher egg ARA levels and dry-weight as well as improved larval survival for the high ARA-DHA diet;
- Low survival during the maternal-to-zygotic transition phase (8 to 16 hpf) impeded larval production, especially for the farm-raised broodstock;
- Larvae from broodstock fed the high ARA-DHA diet with prolonged feeding period reached similar quality as those of their wild-caught counterparts.

Enhancement of EFA, in particular high ARA levels, in the broodstock diets affected the fatty acid composition in the unfertilized eggs and had a positive impact on egg quality and offspring developmental competence. Here, the diet with high ARA and DHA and intermediate EPA levels increased the total lipid content of eggs, the amount of floating eggs, fertilization success, and embryonic survival. In the closely related Japanese eel, studies have shown that too high ARA levels (4.6 – 5.6 % of total FA in the unfertilized eggs) can be detrimental to offspring performance (Furuita et al., 2006; 2007). However, ARA levels between 2.8 and 4.0 % of total FA in the unfertilized eggs characterized the high quality category in Japanese eel and represented our highest levels (3.3 % of total FA in unfertilized eggs). This indicates that the upper limit of ARA levels was not reached in the present study, whilst tested levels promoted offspring developmental competence and larval survival in European eel similar to their Japanese counterparts. These results confirmed the importance of modifying EFA levels in the broodstock diets and added significant new insight to findings of previous qualitative studies limited by offspring availability (Støttrup et al., 2013; 2016). Unprecedented in eel, prolonged feeding with further enhanced diets resulted, in spite slower growth, in higher egg dry-weight and improved larval survival. Thus, results of this study indicate that a long feeding duration with enhanced diets takes precedence over fast growth in relation to reproduction. Such trade-offs in allocation of resources to growth and reproduction need to be considered for future rearing and selection of broodstock.

The study also provided new insights into effects of maternal dietary EFA on embryonic and larval development, resource utilization, and survival. In particular, the analysis of offspring performance related significant bottlenecks in survival of offspring to cleavage

abnormalities and developmental failure during the period of MZT between 8 and 16 hpf, especially for farm-raised females. Using females of wild-caught origin to benchmark results, the study showed a lower frequency of cleavage abnormalities, higher survival during embryonic development, and better hatch rates for offspring obtained from wild-caught females compared to offspring form the best performing diet. Nonetheless, farm-raised female broodstock fed enhanced diets for the longest period of time produced larvae of comparable quality than wild-caught females. These results, which are the first of its kind, are promising for improvement of farm-raised broodstock through enhancement of maternal nutrition in European eel.

Noticeable, levels of EFA were lower in unfertilized eggs from wild-caught females than in those from the best performing diet and feeding regime. This result indicates that although EFA levels in diets may be further enhanced, differences in egg and offspring quality may have additional causes than fatty acids levels in broodstock diets alone. Besides other nutritional aspects, higher occurrence of cleavage abnormalities and mortality during MZT may be related to differences in the endocrinological and developmental state of the farm-raised females at the time of onset of hormonal therapy, affecting responsiveness to the treatment. Thus, pre-treatment administrating E2 in the feed of juvenile eels has been developed for Japanese eel to synchronize ovarian development (Okamura et al., 2014; Tanaka, 2015). Another or additional pre-treatment focuses on supplementation of androgens, such as 11-kt, prior to induction of vitellogenesis to stimulate oocyte development and silvering-related changes to enhance responsiveness to gonadotropic treatment (Di Biase et al., 2017; Lokman et al., 2015; Mordenti et al., 2018; Sudo et al., 2012). We speculated, whether potential differences observed in response to CPE and SPE, possibly related to differences in FSH and LH content, additionally, would influence reproductive success of European eel.

Study 2 Differential impacts of carp and salmon pituitary extracts on induced oogenesis, egg quality, molecular ontogeny and embryonic developmental competence in European eel

This study generated novel insights into eel embryogenesis comparing effects of PEs from two different sources on reproductive success and associating differences in mRNA transcript abundance of targeted genes with quality parameters throughout development.

In order to initiate vitellogenesis in captivity for European eel, application of hormonal treatments in terms of exogenous gonadotropins are required. However, such hormonal manipulations may influence egg and offspring quality in various ways (Mylonas et al., 2010). This might be particularly relevant for eels that receive treatment over a period of 10 weeks or more to reach the spawning stage. During oocyte development, maternal products, including mRNA transcripts, are deposited into the egg, controlling early embryonic development (Abrams and Mullins, 2009; Lubzens et al., 2017; Sullivan et al.,

2015). During the mid-blastula transition, the MZT takes place and developmental control is taken over by the embryos own genome (Newport and Kirschner, 1982; Tadros and Lipshitz, 2009). The successful transition includes the activation of the zygotic gene transcription and the clearance of maternal mRNA, a process that is crucial for successful embryogenesis (Giraldez, 2010; Giraldez et al., 2006; Lee et al., 2014; Schier, 2007; Stitzel and Seydoux, 2007).

The study applied two hormonal treatments, CPE and SPE, administered at a constant dose to induce oocyte maturation comparing their impact on egg quality and offspring developmental competence. Morphological progression was closely monitored in terms of embryonic survival and the occurrence of cleavage abnormalities, while samples were obtained for gene expression analysis. Here, the mRNA transcript abundance of 20 genes important for early teleost development were measured in ovaries after spawning, unfertilized eggs, and embryos throughout development.

Highlights of the study:

- Pituitary extracts from both carp (CPE) and salmon (SPE) induced oogenesis and led to viable larval production;
- Floating egg proportions and embryonic survival were higher and occurrence of cleavage abnormalities lower in offspring from SPE treated females;
- Differential expression patterns throughout embryonic development were observed for 20 genes involved in key mechanisms during early development;
- Expression of 12 of these genes was associated with cleavage abnormalities, embryonic survival or hatch success;
- Higher embryonic survival for SPE treated females was linked to differential expression in eight genes involved in cell adhesion, cell division, cell cycle control, maternal-to-zygotic transition (MZT) activation, and immune regulation.

Overall, both CPE and SPE induced oogenesis and led to viable egg and larvae production. Nonetheless, the gonadotropin types used to induce vitellogenesis affected the reproductive success and offspring quality. While there was a trend of higher responsiveness towards hormonal treatment in the CPE females, offspring obtained from SPE females had higher amounts of floating eggs, lower occurrence of cleavage abnormalities, and higher embryonic survival. Cleavage abnormalities were clearly linked to later embryonic survival explaining the occurring mortality during development. These results may be associated with differences in abundance of mRNA transcripts of genes in the ovary, unfertilized eggs, and embryos.

In this respect, the study is the first to comprehensively analyze mRNA transcript abundance of genes related to embryogenesis throughout development of European eel. Here, differential expression patterns were seen indicating varying importance of specific genes during different stages of the development. Clear patterns were observed indicating maternal effect genes with high mRNA abundance during early embryonic stages and

decreasing levels during the MZT. On the other hand, genes that appear to be of importance during later embryonic development showed lower initial mRNA abundance and distinct increases in expression after activation of the embryos own genome.

Also, a strong family effect with mRNA abundances in unfertilized eggs reflecting levels in the ovary of the females after spawning were found for seven and 13 out of 20 genes for CPE and SPE, respectively. Moreover, the mRNA transcript levels of 12 genes was linked to the occurrence of cleavage abnormalities, embryonic survival, or hatch success. This indicates an important function of these genes for developmental competence in European eel embryos and calls for more dedicated research.

The discrepancies in embryonic survival and cleavage abnormalities between treatments were associated with differential expression of eight genes (*cdhr2*, *cldng*, *dcbld1*, *epcam*, *foxr1*, *ccnb1*, *oct4*, *igm*), which are involved in embryogenesis through processes such as cell adhesion, cell division, cell cycle control, activation of MZT, and regulation of the immune response. This result indicated dissimilarities in the PE extracts affecting the incorporation of maternal mRNA of important genes into the egg, follicular maturation, and embryonic developmental competence. This may be related to differential levels of FSH and LH in the gonadotropin extracts and their distinct importance throughout vitellogenesis and oocyte maturation (Schmitz et al., 2005; Yaron et al., 2003). While this study shows the influence of hormonal treatments to stimulate vitellogenesis, the induction of follicular maturation through administration of MIS may have further impacts on the transfer of maternal products, egg quality, and embryonic developmental competence.

Study 3 Sex steroid dynamics and mRNA transcript profiles of growth and development related genes during embryogenesis following induced follicular maturation in European eel

This study created novel information on sex steroid dynamics and mRNA transcript profiles throughout embryonic development including maternal transfer of sex steroids via the egg to the embryo and their impact on embryogenesis.

Sex steroids, in particular E2 and androgens, are key players in fish reproductive endocrinology and their dynamics throughout maturation have been studied also for anguillid species (e.g. Burgerhout et al., 2016; da Silva et al., 2016; Kazeto et al., 2011). While these hormones naturally are deposited into the egg and likely control early development in teleost fish (Lubzens et al., 2010; 2017; Tokarz et al., 2015), the induction of follicular maturation using PE and DHP administration may lead to unintended transfer impacting viable offspring production.

To measure steroid concentrations and embryonic mRNA transcripts, this study induced vitellogenesis through constant SPE dosage, while follicular maturation was induced using oocyte development as biomarker, a primer dose of SPE, and administration of DHP as

MIS. Using blood plasma samples of the ovary after stripping as well as unfertilized eggs and embryos throughout development, concentrations of E2, T, 11-kt, DHP, and cortisol were measured via radioimmunoassay. Moreover, using similar samples, the mRNA transcript abundance of 15 genes involved in stress response, somatotropic axis, as well as lipid and thyroid metabolism was quantified. To evaluate the effects of steroid levels and mRNA transcripts on the embryos, offspring was categorized into high, medium, and low quality groups based on a combination of fertilization success, cleavage abnormality, embryonic survival at 48 hpf, and hatch success.

Highlights of the study:

- High concentrations of maternally derived DHP and E2 in eggs and embryos negatively impacted the developmental competence of European eel;
- Maternal blood plasma steroid concentrations explained variations of concentrations in the unfertilized eggs for DHP, E2, and T;
- Transcriptional expression profiles of growth, development and metabolism related genes revealed increasing expression after the maternal-to-zygotic transition for the majority of genes;
- Relative expression of *dio3* and *thrαb* was associated with steroid concentrations indicating crosstalk between thyroid hormones and steroids.

All of the five sex steroids analyzed were maternally transferred and present in the unfertilized eggs. More so, concentrations in the maternal blood plasma explained variations in the egg concentrations for DHP, E2, and T indicating a strong maternal effect. On the contrary, concentrations of T, 11-kt, and cortisol were close to detection limits in embryos after fertilization and had no impact on their developmental competence. Although concentrations of DHP and E2 decreased gradually throughout embryogenesis, they significantly affected the ontogeny of the embryos. Here, high concentrations had a negative impact on the developmental capacity in particular after fertilization. Thus, the lack of metabolic degradation of maternally inherited steroids appears to hamper normal development in European eel embryos. This is in line with results from Coho salmon, where DHP and E2 concentrations were higher in non-viable than in viable eggs (Feist et al., 1990). Moreover, unpublished in vitro studies show that European eel ovarian follicles are able to metabolize DHP into inactive DHP-sulphate (H. Tveiten, pers. comm.), which is also known from other teleost species (Scott et al., 1997; Scott and Sorensen, 1994; Tveiten et al., 2000; 2010). This inactivation mechanism is likely to protect the oocyte from DHP overexposure. However, results from this study with high DHP plasma concentrations lead to the speculation that the system may be supersaturated through the DHP injection at extra physiological levels. It can also not be excluded that eggs are forced to enter follicular maturation at too early stages of development, thereby affecting egg quality. At the same time, PE primer of FSH may unintended promote follicular production of E2 negatively affecting egg quality.

These results were supported by mRNA transcript profiles for genes involved in stress response, growth, development and metabolism, which are assumed to be highly important for embryogenesis. Here, the majority of mRNA transcripts appeared to be low during early embryogenesis but showed strong increases in their abundance after the MZT and the embryos own genome activation indicating their importance for later embryonic development. Three genes involved in lipid and thyroid metabolism showed differential expression between quality groups. Here, *cpt1b* was more abundant in high and medium quality groups compared to the low quality one, indicating possible importance for embryogenesis. This is in accordance with a previous study on European eel (Rozenfeld et al., 2016) as well as in Atlantic cod (Lanes et al., 2013), where high quality embryos showed higher relative abundance of this gene. mRNA abundance of *dio1*, involved in thyroid metabolism converting T4 (thyroxine) to T3 (3,5,30-triiodothyronine) was also higher in embryos of high and medium quality compared to those with low developmental potential indicating that the abundance may be of importance during early embryogenesis. The expression of *dio2* and *dio3* showed a pronounced peak towards hatch, which is in line with results from European eel larvae, where elevated levels of these two genes were observed in larvae at hatch (Politis et al., 2018b). Together, these findings may indicate that the thyroid hormone system is functional already during early stages of eel embryogenesis. The study, moreover, observed an association between E2 concentrations and *dio3* expression as well as between T and *thrαb* in the unfertilized eggs indicating a possible interplay between the two hormone systems through receptor binding, which remains to be further investigated.

As such, this second study on influences of assisted reproduction on egg quality and offspring viability has documented maternal transfer of steroids and mRNA transcripts to eggs and embryos in European eel. While steroid levels decrease throughout embryonic development, high levels of maternally derived DHP and E2 related to induced follicular maturation may negatively influence the developmental competence of the embryos. The metabolization of innate steroids appears to be of importance for normal development, though the molecular mechanisms mediating these effects are unclear.

5. Conclusions and future perspectives

This PhD project has provided new insights and substantiated knowledge about female broodstock parameters affecting egg quality, embryogenesis, and early larval ontogeny taking advantage of recent progress in production of offspring that allows quantification of offspring characteristics. The studies have focused on three important aspects related to captive breeding of European eel, which include female nutrition and hormonal treatments and their impact on egg quality and offspring developmental competence. Here, the acquired knowledge has improved our understanding of morphological as well as molecular mechanisms progressing this field of research for captive fish breeding in general and in particular for eel. Insights into the reproductive and molecular levels are essential in order to optimize treatments and protocols as well as developing new therapeutic agents aiming at high quality egg production of marine species in aquaculture.

In Study 1, enhancement of EFA composition in farm-raised female broodstock diets led to improved offspring quality and considering levels of wild-caught female silver eels, we may be approaching optimum levels for dietary requirements of these fatty acids. On the one hand, EPA and DHA levels were not affected by the enhancement and prolonged feeding, indicating that they might have been fed in excess. On the other hand, levels of ARA were highest both, in the diet and eggs that led to the best embryo and larval quality for the farm-raised eels. While future studies may explore benefits of varying EPA/ARA ratios and potentially examine higher ARA levels, alternative enrichment, e.g. vitamins or minerals, might further optimize broodstock nutrition. Another aspect of interest explored in this study indicated that fast-growth may not be ideal for incorporation of nutrients in female eel broodstock. In this regard, the study is extraordinary due to the size-matched broodstock of all three experiments. However, further experiments separating supply of EFA from feeding duration would be needed to separate effects of feeding duration and EFA dietary levels. A major step forward in developing broodstock feeds for eel was the production of larvae from farm-raised females of comparable quality as those from wild-caught females, which is a promising result for future closed-cycle production. Together, the results indicated that the higher developmental success and survival of embryos from wild-caught females may not exclusively relate to broodstock nutrition, as ARA levels in eggs from wild-caught broodstock was lower than in those from farm-raised broodstock.

The study also defined a bottleneck during embryonic development affecting survival around the MZT. Resolving the causes and overcoming this obstacle can enhance offspring production and increase efficiency producing larvae from farm-raised broodstock using wild-caught broodstock as benchmark. Differences in embryonic survival may refer to a better responsiveness of wild-caught females due to a more advanced endocrinological and morphological state at the onset of hormonal therapy. Here, feminization procedures through feed intake or pre-treatment with androgens such as 11-kt for farm-raised females may stimulate oocyte development with possible improvements in embryonic developmental competence. Furthermore differences may relate to the

pituitary extracts used in assisted reproduction of European eel in relation to treatment over a longer period of time. Presently, techniques such as ELISA to test hormone composition in PEs are not available, however, first insight into the efficacy CPE and SPE might guide the way to improved treatments.

The results of Study 2 emphasized differences in the effect of CPE and SPE on responsiveness and in particular egg and embryonic quality. As anticipated, both treatments resulted in the production of viable offspring. However, the impact on the production of floating eggs and thereby viable egg production as well as cleavage abnormalities and embryonic survival differed significantly. Here, cleavage abnormalities explained the occurring mortality during later embryonic development. Moreover, the lack of cell adhesion, as well as embryonic survival, and hatch success were related to abundance of specific maternal mRNA transcripts incorporated in the oocytes during vitellogenesis. The abundance of mRNA transcripts also differed between treatments indicating that dissimilarities in the PE extracts affected the incorporation of maternal mRNA of important genes into the egg, as well as follicular maturation and embryonic developmental competence. We reason that these differences partially relate to differences in FSH and LH levels in the pituitaries that may vary depending on the species and the reproductive state of the fish from which the pituitaries originate. Here, a better understanding of FSH and LH levels in pituitaries through development of new tools, such as ELISA may prove useful, as shown in recent studies on Japanese eel (Kazeto et al., 2019). Developing therapy and treatment schemes using recombinant gonadotropins would benefit from insights in the endocrine regulation and requirements for FSH and LH during vitellogenesis and follicular maturation. Here, first promising results have been shown for Japanese female eels (Kazeto et al., 2019), as well as European male eels (Herranz-Jusdado et al., 2019b; Peñaranda et al., 2018).

Results of Study 3 documented the maternal transfer of sex steroids to the egg in relation to induction of follicular maturation in European eel. Moreover, high levels of DHP and E2 in the eggs and embryos negatively impacted the developmental competence. This may be related to inducing follicular maturation with a priming dose of PE and subsequent administration of MIS, leading to unnaturally high concentrations that influence the transfer to the offspring. Here, this thesis showed that follicular maturation represents a critical step in the assisted reproduction procedures that impacts egg quality and subsequent development of the embryos. Nonetheless, the molecular mechanisms behind these varying steroid concentrations remain to be investigated and potential studies may examine the inactivation mechanisms that metabolize DHP into inactive DHP-sulphate to avoid possible supersaturation and recruitment of eggs at too early stage of development. Surprisingly, despite assisted reproduction procedures, concentrations of transferred cortisol were low and had no implications on embryonic development. This may indicate that eels possess protective measures to prevent excess cortisol entering the eggs, such as upregulation of cortisol inactivating enzymes (11β-hydroxysteroid dehydrogenase type 2). Future studies may investigate, whether a similar mechanism exists in European eel

ovaries in order to protect the offspring. Study 3 moreover elucidated expression patterns of genes involved in growth and metabolism and their effect on embryonic development. Here, the study indicates the thyroid system may already be functional during early embryonic development and further revealed a possible crosstalk between the steroid and thyroid hormone systems in eggs and embryos of European eel. A combined analysis of steroid and thyroid hormones as well as their receptors and converting enzymes to elucidate their precise function and role during early teleost development would be of interest.

Molecular tools in combination with traditional tools such as fatty acid analysis and radioimmunoassay of hormones increased our insights into underlying processes and mechanisms related to morphological findings. Together, the studies of this thesis have deepened our understanding of aspects critical to production of progeny and their survival during early life history through the application and combination of these tools. In particular, the molecular analyses documented the maternal transfer of mRNA transcripts of important maternal effect genes influencing the developmental competence of the embryos at early stages. Moreover, analyses defined expression patterns throughout embryogenesis with particular focus on the MZT. Here, patterns of maternal effect genes showed high abundance of mRNA transcripts in the unfertilized eggs and early stages with decreasing levels during the MZT. The majority of genes involved in stress response, growth and development as well as lipid and thyroid metabolism showed an increased expression after the embryos own genome was activated indicating importance for later embryonic stages. Here, the PhD project has underlined the importance of successful transition during MZT for European eel to obtain healthy embryos and larvae. It would be interesting to advance present findings and similar results using transcriptomic analyses to overview the whole genome transcription in the unfertilized egg as well as in developing embryos. This would allow further detection of maternal mRNA transcripts and expression of genes that are essential for normal development.

In conclusion, results of the studies of this thesis have contributed to gain insights into requirements for obtaining eggs and larvae of high quality by adapting broodstock nutrition and developing hormonal treatments. Altogether, the studies strengthens the assumptions that it is feasible to produce egg and larvae of a quality needed for an efficient hatchery production of European eel.

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Manuscripts

Paper I: 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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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; cleavage abnormalities

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; Labbe and Maisse, 2001; Shields et al., 1997), 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., 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., 2007, 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 approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696). Individual fish were anesthetized before tagging, biopsy, and stripping of gametes, and euthanized after stripping (females) or at the end of the experiment (males) using an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg L^{-1}, Sigma Aldrich,

Germany). Larvae were anesthetized and euthanized using tricaine methanesulfonate (MS-222, 25 mg L^{-1}, Sigma Aldrich, Germany).

2.2 Fish and experimental design

2.2.1 **Experimental overview**

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

2.2.2 **Diets**

Broodstock diets were formulated with the purpose to generate three dietary regimes by modifying levels and ratios of ARA, EPA, and DHA in eggs and yolk sac larvae. Diet 1 aimed at the highest levels of ARA and DHA and intermediate EPA levels. Diet 2 comprised the lowest ARA level, but the highest EPA and intermediate DHA levels. Therefore, the 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). In the second production, while maintaining similar levels and differences in composition of ARA, EPA, and DHA, 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. Additionally, DHA

Liquid replaced the EPAX, to balance differences in PUFA. From each production, one feed sample per diet was taken at the onset of feeding, and subsequently analyzed for fatty acid composition.

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) 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 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 (Butts et al., 2014; 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 (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 hatch 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. 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 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. Lipids from feed samples were extracted and esterified to methyl esters (Støttrup et al., 2013) and fatty acids were measured as above.

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 the effect of dietary regime and feeding trial on embryonic survival and Model 7 tested parameters characterizing embryonic development. The effect of broodstock origin on the same traits was tested in Model 6 and 8 (Table 2). Moreover, a linear regression function was used to analyze the relationship between

cleavage abnormalities at 4 hpf and embryonic survival at 48 hpf. Due to low numbers of hatched larvae, insufficient larval data were obtained for Diet 2 and 3. Therefore, only the effect of broodstock origin on larval survival and morphology was tested in Models 9 and 10, respectively (Table 2).

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

3 Results

3.1 Diets and broodstock

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

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.

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 ($p = 0.161$; 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 ($p = 0.006$).

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 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. 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 intermediate (Fig. 1A; Model 3). On the other hand, total lipid content was similar for females from Trial 1 and Trial 2 ($p = 0.486$; Fig. 1B). Moreover, total lipid did not differ among unfertilized eggs of Diet 1 Trial 1 and Trial 2, and wild-caught females ($p = 0.495$; Fig. 1C; Model 4). Similarly, total lipid content did not differ between unfertilized eggs and larvae at 0 dph, while total lipid of larvae at 5 dph was significantly lower than in the eggs and in newly hatched larvae ($p < 0.0001$; Fig. 1D).

3.4 Fatty acid composition in eggs and larvae

Unfertilized eggs reflected dietary regime ($p < 0.0001$), such that eggs of females reared on Diet 1 had the highest relative ARA levels and those of Diet 2 the lowest (Fig. 2A; Model 3). Notably, eggs obtained from females of Trial 2 had higher ARA levels than those of Trial 1 ($p = 0.007$; Fig. 2B). Similarly, dietary regime ($p = 0.012$) affected EPA levels of unfertilized eggs. Here, eggs from females reared on Diet 2 showed higher EPA levels than eggs from those reared on Diet 3, while values for eggs obtained from females fed Diet 1 were intermediate (Fig. 2C). Here, EPA levels of eggs from Trial 2 females were lower than those from Trial 1 females ($p = 0.040$; Fig. 2D). Moreover, DHA levels of unfertilized eggs differed between the dietary regimes ($p = 0.006$), such that those from females reared on Diet 2 had higher DHA

content than those obtained from Diet 1 or Diet 3 (Fig. 2E), while eggs from Trial 1 and 2 females did not differ in this respect ($p = 0.163$; Fig. 2F). The relative fatty acid content of unfertilized eggs from the seven groups of females is given in Table A.3.

The relative ARA levels of unfertilized eggs and larvae also differed among Diet 1 Trial 1, Diet 1 Trial 2, wild-caught broodstock. Here, the highest ARA levels related to eggs from Trial 2 females reared on Diet 1, and the lowest to wild-caught female eggs ($p < 0.0001$; Fig. 2G; Model 4). The ARA levels were similar in unfertilized eggs and larvae at 0 dph, but relatively higher in larvae at 5 dph ($p < 0.0001$; Fig. 2H). Similarly, EPA levels were higher in eggs and offspring of females reared on Diet 1 compared to those of wild-caught ($p < 0.0001$; Fig. 2I), while life stage, i.e. unfertilized eggs and larvae at 0 and 5 dph, did not affect the relative content ($p = 0.287$; Fig. 2J). Furthermore, DHA levels in eggs and larvae depended on broodstock with the highest values relating to Trial 1 females fed Diet 1 and the lowest to wild-caught females ($p < 0.0001$; Fig. 2K). DHA levels were similar for unfertilized eggs and hatched larvae, while the relative content was higher at 5 dph ($p < 0.0001$; Fig. 2L). The relative fatty acid composition of larvae at 0 and 5 dph from the three groups of females is given in Table A.4. Overall, eggs and larvae of farm-raised females fed Diet 1 showed higher amounts of PUFA, while certain saturated fatty acids and MUFA levels were lower than in those of wild-caught females (Tables A.3 and A.4). For instance, the levels of palmitoleic acid, 16:1 (n-7), oleic acid, 18-1 (n-9), and cis-vaccenic acid, 18-1 (n-7), were consistently lower in eggs as well as larvae at 0 and 5 dph in offspring from farm-raised females fed Diet 1 compared to those of wild-caught females. During the first 5 dph, saturated fatty acid and MUFA levels of Diet 1 Trial 1 and 2, as well as wild-caught females, decreased slightly, while PUFA levels increased in the percentage of total fatty acids. The sum of n-3 and n-6 fatty acids were higher in eggs and larvae obtained from farm-raised females fed Diet 1, showing a higher n-6 to n-3 ratio compared to those of wild-caught. Comparing eggs of farm-raised females, eggs obtained

from females fed Diet 2 had the lowest n-6 to n-3 ratio. The EPA:ARA ratio was lowest in Diet 1, in particular Diet 1 Trial 2, and highest in Diet 2. Throughout life stages, i.e. unfertilized eggs, larvae at 0 and 5 dph, the EPA:ARA was similar between wild-caught and Diet 1 offspring.

3.5 Fertilization success

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

3.6 Embryonic development

3.6.1 Survival

Similar to fertilization success, embryonic survival differed between the three dietary regimes, such that embryos obtained from females reared on Diet 1 showed the highest survival ($p < 0.0001$; Fig. 4A; Model 5), while no effect of feeding trial on embryonic survival was detected ($p = 0.902$; Fig. 4B). During embryonic development, the survival rate declined slightly from 4 to 8 hpf, followed by a steep decline from 8 to 16 hpf after which survival stabilized ($p < 0.0001$; Fig. 4C). Furthermore, embryonic survival varied considerably among offspring from individual females in particular for Diet 3 (Fig. 4D-F).

Furthermore, embryonic survival was lower for Diet 1 females than for wild-caught females ($p < 0.001$; Fig. 4G; Model 6). As above, embryonic mortality was highest in the early stages and stabilized thereafter ($p < 0.0001$; Fig. 4H). The variability among offspring from

individual females was high for embryonic survival, especially for the wild-caught broodstock (Fig. 4I-K). Here, offspring of farm-raised broodstock fed Diet 1 showed the characteristic decline in survival between 8 and 16 hpf. In contrast, wild-caught broodstock with >50% fertilization success at 4 hpf had consistently higher survival throughout development (Fig. 4K).

3.6.2 **Morphology**

Morphological characteristics of embryos at 4 hpf did not differ among offspring derived from different dietary regimes in terms of egg area (Diet 1: 1.46 ± 0.08 mm^2, Diet 2: 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, 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: 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 7). Neither did these measures differ between feeding trials, i.e. egg area (Trial 1: 1.32 ± 0.10 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 ± 0.01 mm^2; p = 0.333), and oil droplet area (Trial 1: 0.099 ± 0.002 mm^2, Trial 2: 0.103 ± 0.002 mm^2; p = 0.168). Data at 48 hpf were excluded from these analyses as the number of embryos available was insufficient.

Embryonic morphology at 4 hpf also did not differ among broodstock origin in terms of 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 ± 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 ± 0.02 mm^2, wild-caught: 0.65 ± 0.02 mm^2, p = 0.405), and oil droplet area (Diet 1 Trial 1: 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 = 0.144; Model 8). Also, egg area at 48 hpf was similar (Diet 1 Trial 1: 1.53 ± 0.16 mm^2, Diet 1 Trial 2: 1.69 ± 0.16 mm^2, wild-caught: 1.81 ± 0.16, p = 0.446).

3.6.3 Cleavage abnormalities

Cleavage abnormalities at 4 hpf 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. 5A) nor feeding trial was found ($p = 0.121$; Fig. 5B; 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. 5C; Model 8), while Diet 1 Trial 2 did not differ significantly. No significant relationship between abnormalities and survival at 48 hpf was detected for embryos from the farm-raised females fed Diet 1 in Trial 1 (Fig. 5D), while cleavage abnormalities were related to embryonic survival for farm-raised females fed Diet 1 in Trial 2 (Fig. 5E) and wild-caught females (Fig. 5F). When pooling data from all females independent of origin, a highly significant relationship appeared (Fig. 5G).

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. Here, 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.

7A; 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. 7B). 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). Here, 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 2 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

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

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

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

Levels of EPA and DHA in broodstock diets may also affect egg quality and offspring performance. For instance, in gilthead seabream, *Sparus aurata* EPA levels were positively

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.

In the present study, the females that required a prolonged feeding period to reach the same size, while receiving further enhanced diets accumulated more ARA and produced offspring of higher quality, from unfertilized eggs up to the larval stage. It cannot be concluded

from this experiment, if the females accumulated ARA in a more efficient way, however, the trends in selective accumulation of ARA over time was similarly indicated in the studies of Støttrup et al. (2013; 2016). Thus, females with slower growth performance throughout the feeding trials, on average needed an additional 24 wks of feeding to meet the same size criteria.

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

The size-matched females fed enhanced diets over the prolonged feeding period furthermore produced eggs of higher dry weight. Egg size and dry weight are often influenced by maternal size, and used as quality indicators as they affect early life history in marine fish (Bobe and Labbé, 2010; Kjørsvik et al., 1990; Rideout et al., 2005; Trippel, 1998). For instance, in Atlantic cod, egg dry weight and fecundity was lower in poor condition females (Lambert and Dutil, 2000; Ouellet et al., 2001). On the other hand, egg dry-weight was negatively correlated to cell clarity (Penney et al., 2006). Previous studies on European eel did not find any relation between dry-weight and offspring quality (da Silva et al., 2018; Rozenfeld et al., 2016). However, the results of this more comprehensive study indicate that dry weight might

play a role in defining embryonic developmental competence and thus dry weight in combination with EFA levels may prove useful as quality indicators in European eel.

In accordance with these egg quality parameters, the larvae obtained from farm-raised broodstock fed Diet 1 with prolonged feeding reached similar quality as those of their wild-caught counterparts. Notably, once hatched larval survival was comparable between wild-caught and farm-raised females fed Diet 1 for the prolonged feeding period, and the body area of larvae from these farm-raised females was even significantly higher than of those from wild-caught females. The study further revealed a selective retention of ARA and DHA during early larval development reflecting their importance i.e. in structural development, especially neural and visual functions (Sargent et al., 1999b). This is similar to other studies on Florida pompano, *Trachinotus carolinus*, and common snook, *Centropomus undecimalis* (Hauville et al., 2016), as well as Atlantic bluefin tuna, *Thunnus thynnus* (Morais et al., 2011) where relative levels of ARA and DHA increased during the first 4-6 dph together with utilization of total lipids as an energy source. The effect of EFA levels on larval survival and development appears to be highly species specific. While a positive effect of DHA is widely distributed (Glencross, 2009), the effect of ARA reaches from positive (Mazorra et al., 2003), over neutral (Hauville et al., 2016) to negative effects (Røjbek et al., 2014). In the Atlantic halibut, larval survival was found to be significantly higher from females fed with an ARA enhanced diet (Mazorra et al., 2003), which coincides with our results. In the European eel, overall, PUFAs were preserved in the larvae of the two farmed as well as the wild caught groups, while mainly saturated fatty acids and MUFAs were used during early larval development.

The most prominent difference between the wild-caught and farm-raised broodstock was differences in embryonic survival and hatch success. The lower survival of offspring from farm-raised females was related to a higher percentage of cleavage abnormalities assessed at 4 hpf. Abnormal cleavage patterns have been shown to cause higher embryonic mortality in

Atlantic cod (Avery et al., 2009), yellowtail flounder, *Limanda ferruginea* (Avery and Brown, 2005), and turbot, *Scophthalmus maximus* (Kjørsvik et al., 2003). Also in the present case, the abnormal cleavages affected embryonic development leading to a sharp decline in survival between 8 hpf and 16 hpf, resulting in low hatch success.

This suggest that zygotic and early embryonic development in European eel subjected to assisted reproduction is influenced by more factors than maternal nutrition and resulting egg dry weight, lipid content and fatty acid composition (Mylonas et al. 2010). Here, an important step in embryonic development is characterized by the maternal to zygotic transition (MZT), in which developmental control is taken over by the activation of zygotic transcription (Newport and Kirschner, 1982). This change takes place during the mid-blastula transition, which in European eel occurs at ~10 hpf at 18°C (Sørensen et al., 2016b). Until this point, maternal gene products are the most essential drivers for early embryonic development. Studies have shown essential impacts of the abundance of specific mRNA transcripts on egg quality and embryonic development (Aegerter et al., 2004; Lanes et al., 2013; Rozenfeld et al., 2016; Škugor et al., 2014). The observed decline in survival of embryos from farm-raised females around this time in embryonic development indicates 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 offspring with higher fertilization capacity 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. Notably, 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 present study further improved egg quality and offspring performance originating from farm-raised female European eel broodstock through enhanced diet composition. By following embryonic and larval development, we further documented the importance of egg dry weight, lipid content, and EFA for embryonic and larval development. Thus, adjusting dietary levels of ARA, EPA and DHA enhanced the egg quality and offspring performance significantly in farm-raised broodstock, in particular highlighting the importance of ARA. The results furthermore drew attention to the importance of long feeding duration in eel prior to onset of assisted reproduction. Here, the dietary EFA levels combined with slow growth proved

superior enhancing effects on egg and offspring quality. Furthermore, 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. Here, several factors influence farm-raised broodstock performance and inferior embryonic development competence may besides nutritional factors relate to the endocrinological state of the females at the onset of the induced maturation, i.e. they have not naturally reached the silvering stage. 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. Here, the also two EFAs, ARA and DHA were retained in the yolk sac larvae demonstrating their importance during early ontogeny. Together, findings of this study can be used in future broodstock establishment and reproduction of European eel to improve offspring quality aiming at a closed cycle production in captivity.

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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

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

Table 3. Essential 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.

	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

Table 4. Characteristics of European eel, *Anguilla anguilla* broodstock, eggs, and offspring from females fed on three different diets 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

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

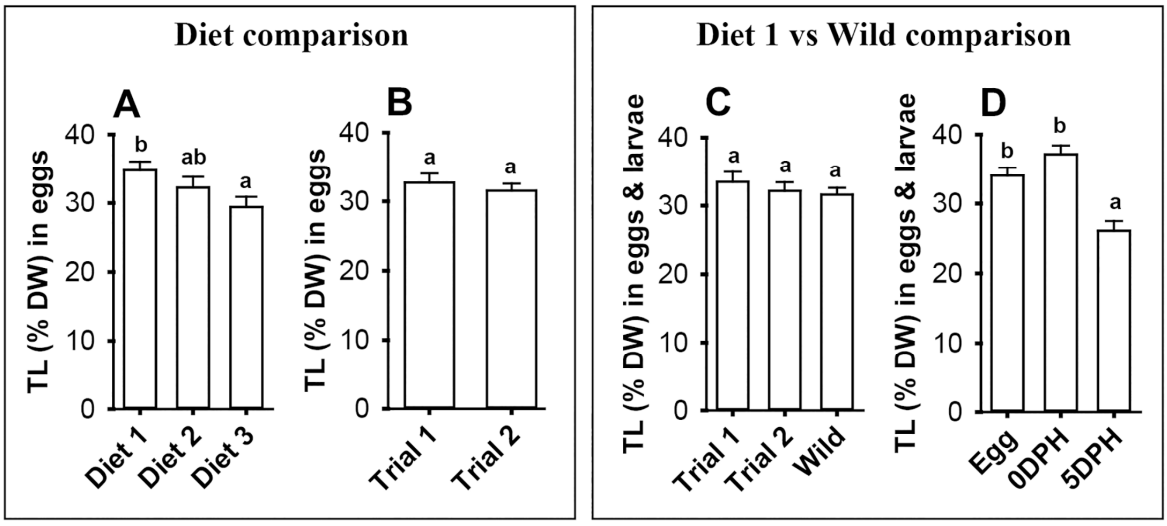

Fig. 1 Total lipid content in eggs and larvae of European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A) and feeding trial (B) on total lipid (TL) in % dry weight (DW) of unfertilized eggs from farm-raised eels (n = 34). Effects of female broodstock origin (Diet 1 Trial 1 and Trial 2; wild-caught fish) (C) and offspring age 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 ($p < 0.05$).

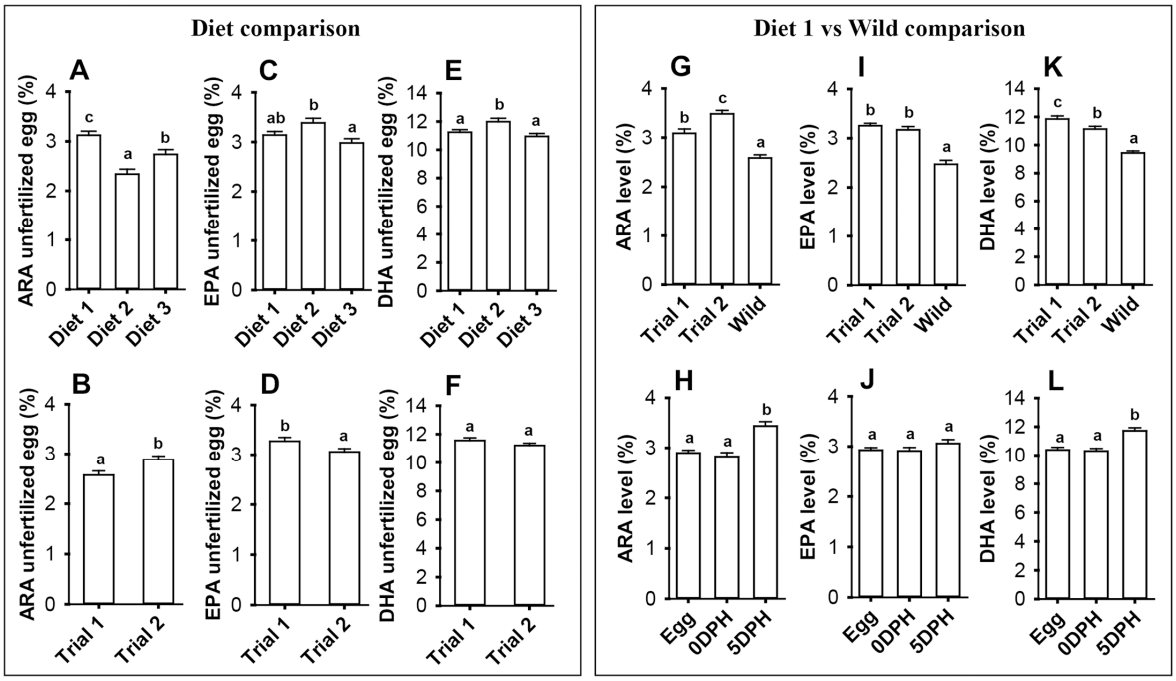

Fig. 2. Relative fatty acid content in unfertilized eggs and larvae of European eel, *Anguilla anguilla*. Effects of maternal dietary regime and feeding trial on unfertilized egg levels (%) of ARA (A, B), EPA (C, D) and DHA (E, F) (n = 33). Effects of broodstock origin and age on eggs and larvae for 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 ($p < 0.05$).

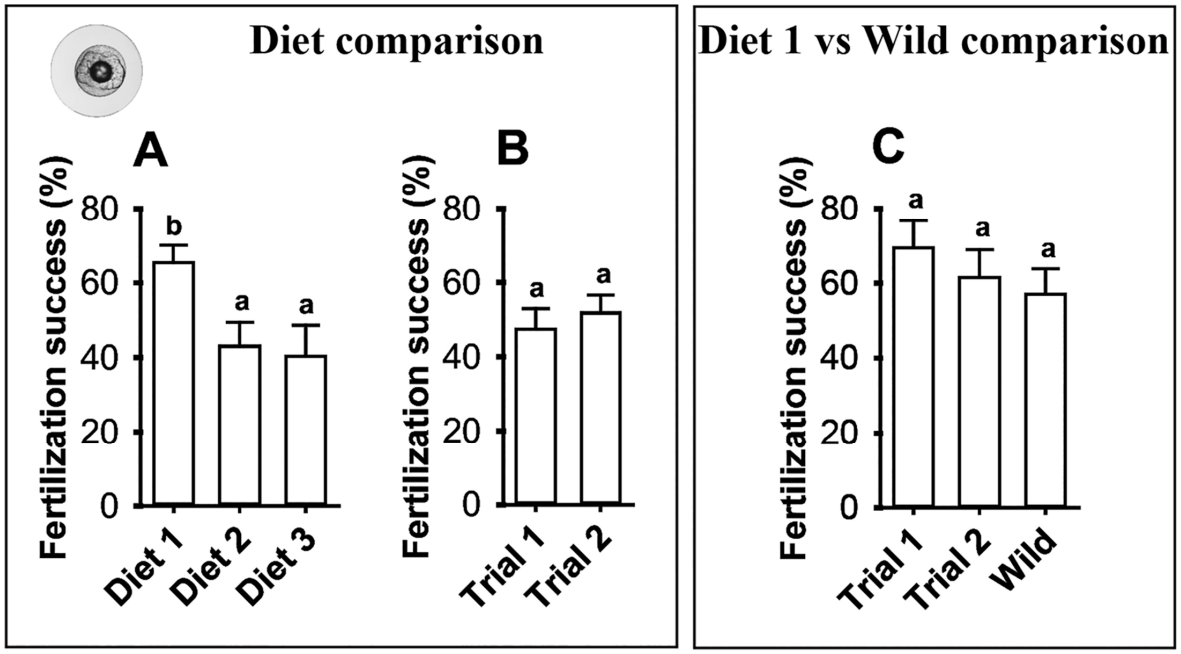

Fig. 3. Fertilization success in European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A), and feeding trial (B) of farm-raised females (n = 23) as well as broodstock origin on fertilization success (C) (n = 19). Values represent means (± SEM) among females at each sampling time and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

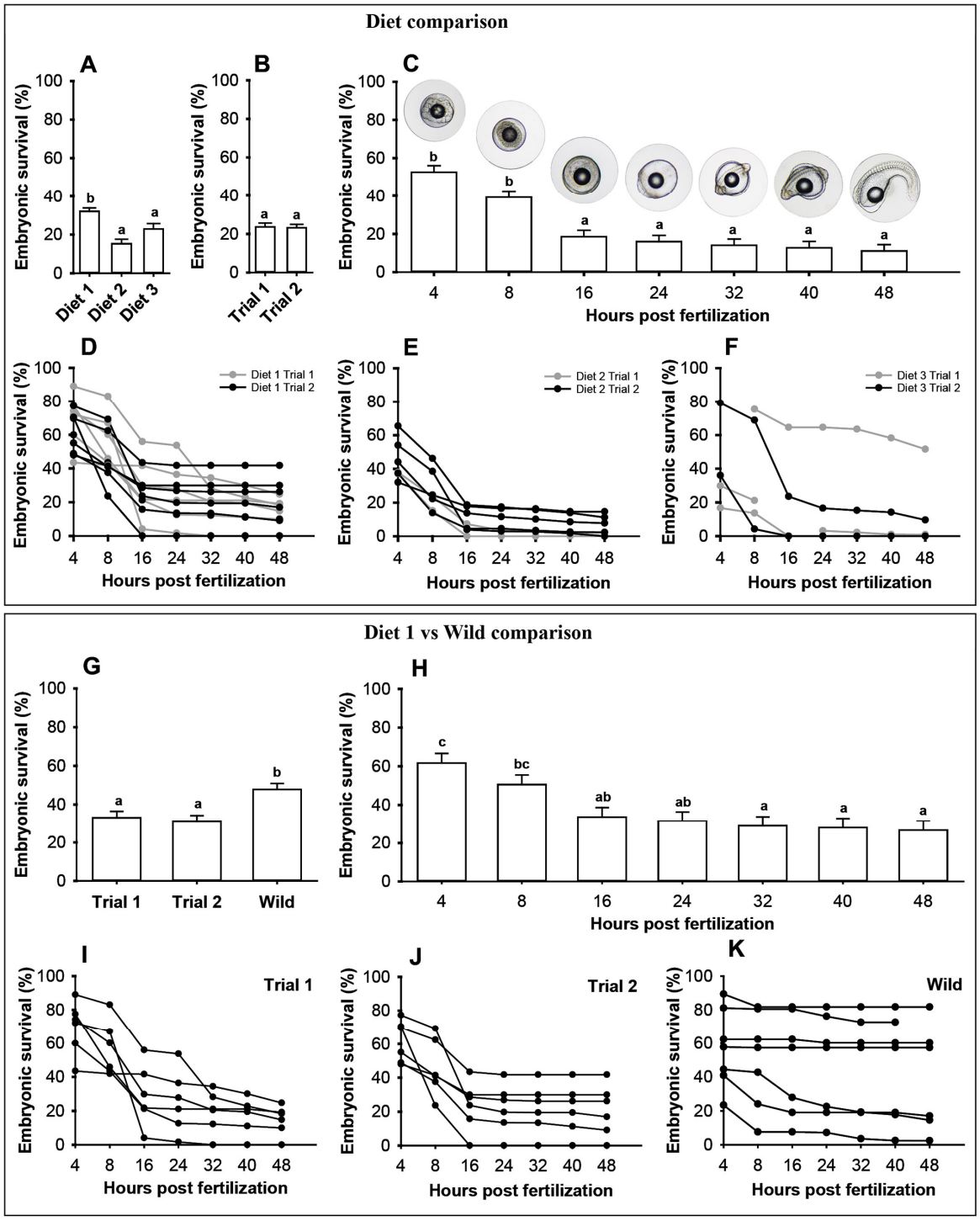

Fig. 4. Embryonic survival in European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A), feeding trial (B), and offspring age (4-48 hours post fertilization; C) on embryonic survival as well as their survival over time for individual females fed Diet 1 (D), Diet 2 (E), and Diet 3 (F) (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 ($p < 0.05$).

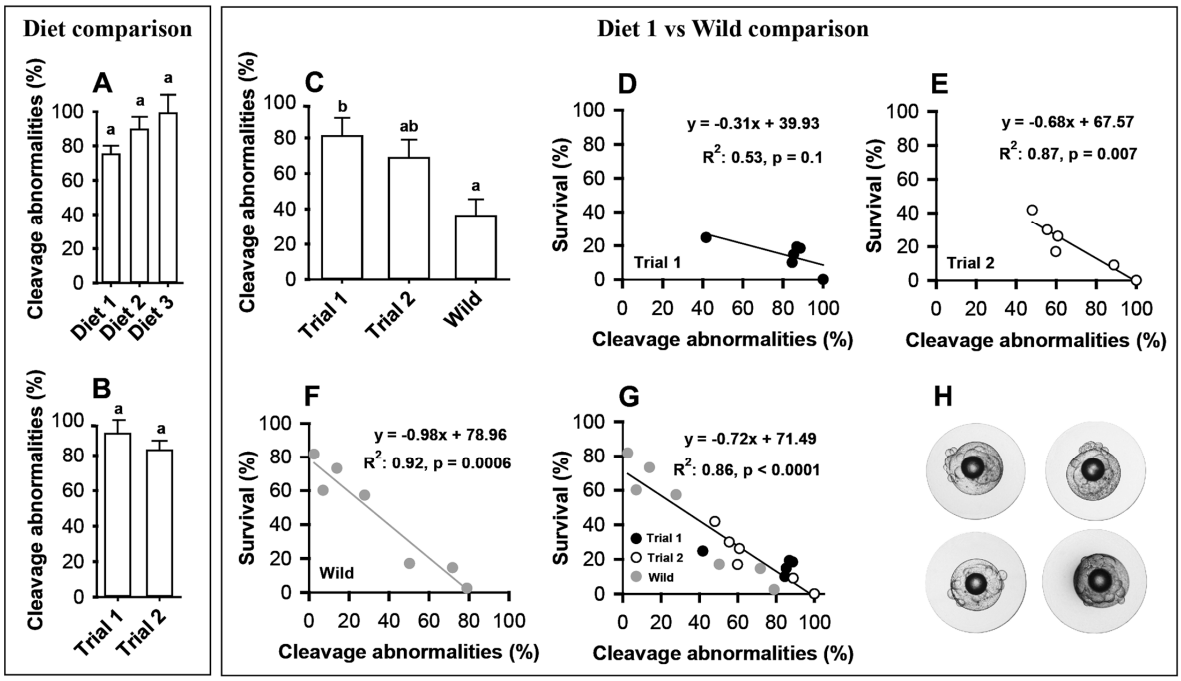

Fig. 5 Cleavage abnormalities in European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A) and feeding trial (B) in farm-raised female eels (n = 22) as well as broodstock origin (C) (n = 19) on proportion of cleavage abnormalities in offspring at 4 hours post fertilization (hpf). Relationships between cleavage abnormalities at 4 hpf and embryonic survival at 48 hpf display offspring of individual females for Diet 1 Trial 1 (D), Diet 1 Trial 2 (E), wild-caught (F), all three treatments pooled (G), and typical abnormal cleavage patterns (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 ($p < 0.05$).

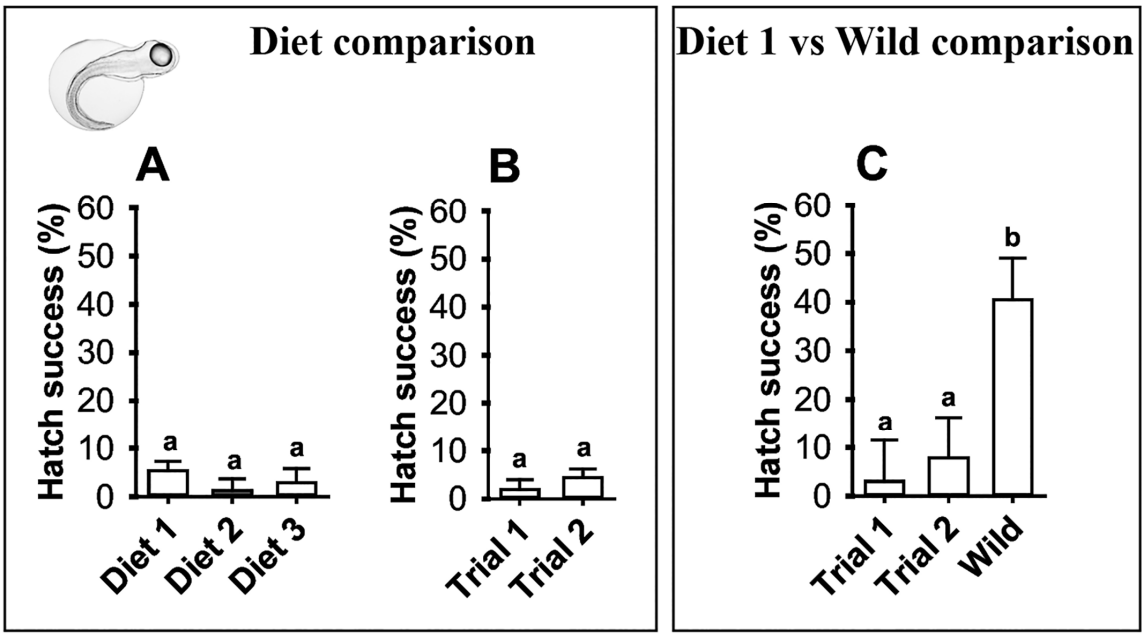

Fig. 6. Hatch success in European eel, *Anguilla anguilla*. Effects of maternal dietary regime (A), feeding trial (B) (n = 24), and broodstock origin (C) (n = 18) on hatch success (%). Values represent means (± SEM) among females at each sampling point and treatment. Different lower-case letters represent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

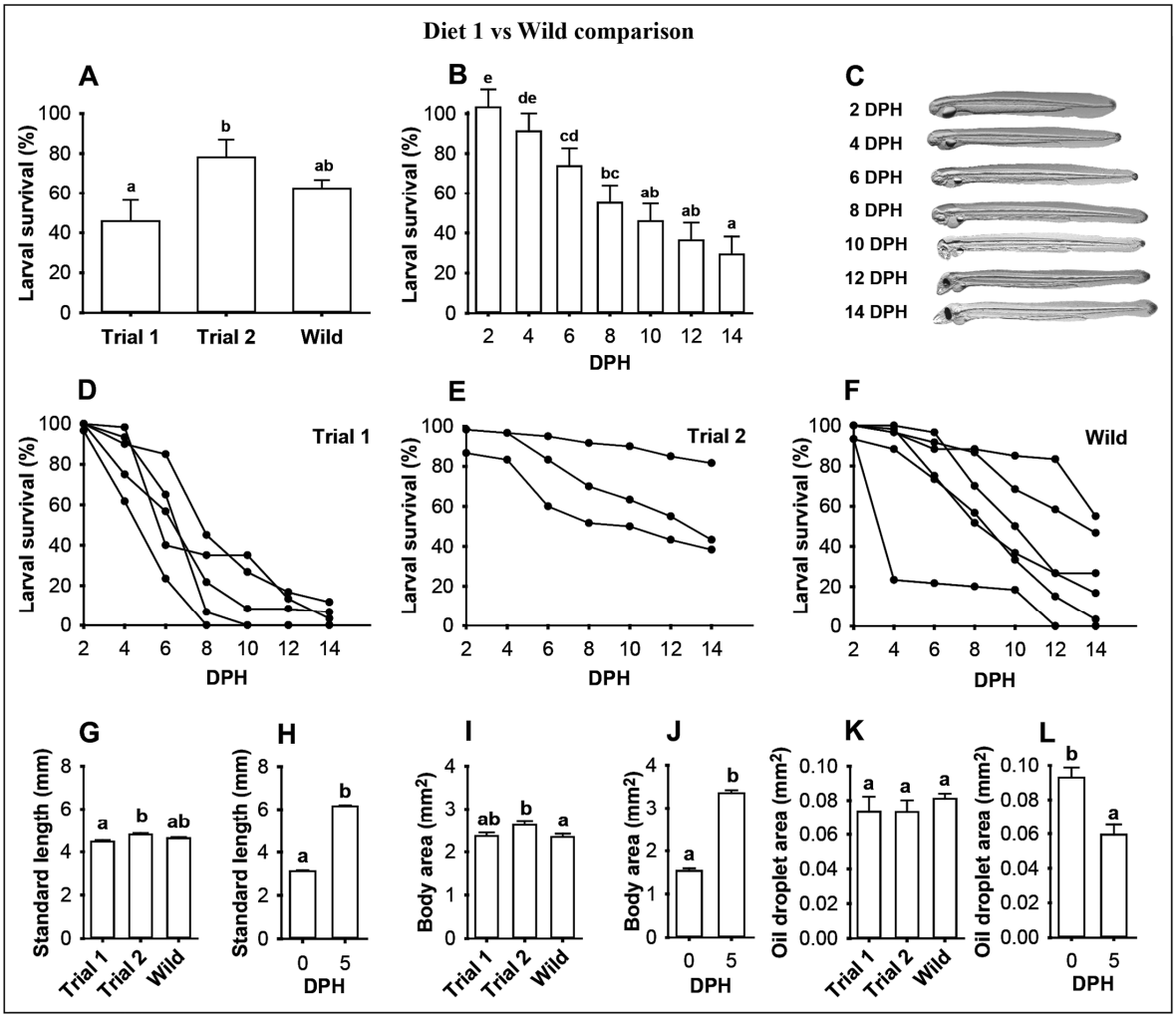

Fig. 7. Larval survival and development in European eel, *Anguilla anguilla*. Effects of broodstock origin (A) and offspring age (days post hatch, dph; B) on larval survival as well as larval survival over time (C) (n = 14). Survival of larvae from individual females in relation to maternal origin is 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 ($p < 0.05$).

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*.

Fig.	Fig. part	Treatment	Sampling	Sample size (n)
1	A, B, C, D	Diet 1 Trial 1	Unfertilized eggs	6
1	A, B, C, D	Diet 1 Trial 2	Unfertilized eggs	8
1	A, B	Diet 2 Trial 1	Unfertilized eggs	3
1	A, B	Diet 2 Trial 2	Unfertilized eggs	6
1	A, B	Diet 3 Trial 1	Unfertilized eggs	4
1	A, B	Diet 3 Trial 2	Unfertilized eggs	7
1	C, D	Wild	Unfertilized eggs	10
1	C, D	Diet 1 Trial 1	0 dph	3
1	C, D	Diet 1 Trial 2	0 dph	4
1	C, D	Wild	0 dph	5
1	C, D	Diet 1 Trial 1	5 dph	2
1	C, D	Diet 1 Trial 2	5 dph	3
1	C, D	Wild	5 dph	5
2	A-F	Diet 1 Trial 1	Unfertilized eggs	6
2	A-F	Diet 1 Trial 2	Unfertilized eggs	8
2	A-F	Diet 2 Trial 1	Unfertilized eggs	3
2	A-F	Diet 2 Trial 2	Unfertilized eggs	6
2	A-F	Diet 3 Trial 1	Unfertilized eggs	4
2	A-F	Diet 3 Trial 2	Unfertilized eggs	6
2	G-L	Diet 1 Trial 1	Unfertilized eggs	6
2	G-L	Diet 1 Trial 2	Unfertilized eggs	8
2	G-L	Wild	Unfertilized eggs	11
2	G-L	Diet 1 Trial 1	0 dph	3
2	G-L	Diet 1 Trial 2	0 dph	4
2	G-L	Wild	0 dph	6
2	G-L	Diet 1 Trial 1	5 dph	2
2	G-L	Diet 1 Trial 2	5 dph	3
2	G-L	Wild	5 dph	5
3	A, B, C	Diet 1 Trial 1	4 hpf	6
3	A, B, C	Diet 1 Trial 2	4 hpf	6
3	A, B	Diet 2 Trial 1	4 hpf	2
3	A, B	Diet 2 Trial 2	4 hpf	5
3	A, B	Diet 3 Trial 1	4 hpf	2
3	A, B	Diet 3 Trial 2	4 hpf	2
3	C	Wild	4 hpf	7
4	A-F, G-K	Diet 1 Trial 1	4-48 hpf	6
4	A-F, G-K	Diet 1 Trial 2	4-48 hpf	6
4	A-F	Diet 2 Trial 1	4-48 hpf	2
4	A-F	Diet 2 Trial 2	4-48 hpf	5
4	A-F	Diet 3 Trial 1	4, 16 hpf	3
4	A-F	Diet 3 Trial 1	8, 24-48 hpf	4
4	A-F	Diet 3 Trial 2	4-48 hpf	3
4	G-K	Wild	4, 24-40 hpf	8
4	G-K	Wild	8, 16, 48 hpf	7
5	A, B, C, D, G	Diet 1 Trial 1	4 hpf	6
5	A, B, C, E, G	Diet 1 Trial 2	4 hpf	6
5	A, B	Diet 2 Trial 1	4 hpf	2
5	A, B	Diet 2 Trial 2	4 hpf	5
5	A, B	Diet 3 Trial 1	4 hpf	1
5	A, B	Diet 3 Trial 2	4 hpf	2
5	C, F, G	Wild	4 hpf	7
6	A, B, C	Diet 1 Trial 1	0 dph	6
6	A, B, C	Diet 1 Trial 2	0 dph	6
6	A, B	Diet 2 Trial 1	0 dph	2
6	A, B	Diet 2 Trial 2	0 dph	5
6	A, B	Diet 3 Trial 1	0 dph	3
6	A, B	Diet 3 Trial 2	0 dph	2
6	C	Wild	0 dph	6
7	A, B, D	Diet 1 Trial 1	2, 4, 6, 8, 10, 12, 14 dph	5
7	A, B, E	Diet 1 Trial 2	2, 4, 6, 8, 10, 12, 14 dph	3
7	A, B, F	Wild	2, 4, 6, 8, 10, 12, 14 dph	6
7	G-L	Diet 1 Trial 1	0 dph	4
7	G-L	Diet 1 Trial 2	0 dph	4
7	G-L	Wild	0 dph	5
7	G-L	Diet 1 Trial 1	5 dph	3
7	G-L	Diet 1 Trial 2	5 dph	3
7	G-L	Wild	5 dph	5

hpf: hours post fertilization; dph: days post hatch

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.

	Diet 1_1	**Diet 1_2**	**Diet 2_1**	**Diet 2_2**	**Diet 3_1**	**Diet 3_2**
Fatty acid						
14:0	4.73±0.13	3.74±0.02	5.09±0.07	4.35±0.03	4.04±0.01	3.95±0.06
15:0	0.24±0.00	0.40±0.01	0.25±0.01	0.42±0.00	0.21±0.00	0.40±0.01
16:0	10.98±0.05	14.37±0.14	11.15±0.12	15.01±0.11	10.27±0.04	14.42±0.04
17:0	2.17±0.03	3.63±0.03	1.81±0.07	3.22±0.02	1.98±0.00	3.35±0.01
18:0	0.23±0.09	0.25±0.00	0.27±0.04	0.32±0.00	0.19±0.08	0.28±0.00
20:0	0.31±0.03	0.31±0.00	0.24±0.00	0.28±0.00	0.35±0.02	0.29±0.01
SUM	**18.65±0.08**	**22.70±0.14**	**18.81±0.08**	**23.6±0.11**	**17.03±0.11**	**22.69±0.04**
14:1	0.09±0.00	0.15±0.00	0.10±0.00	0.17±0.00	0.08±0.00	0.16±0.02
16:1 (n-7)	6.34±0.01	4.32±0.02	6.93±0.08	5.01±0.02	5.59±0.00	4.48±0.03
18:1 (n-9)	11.04±0.01	16.13±0.00	10.53±0.05	14.97±0.06	17.50±0.11	15.89±0.02
18:1 (n-7)	2.54±0.01	2.12±0.01	2.65±0.03	2.27±0.02	2.55±0.00	2.17±0.00
20:1 (n-9,n-11)	11.00±0.07	4.21±0.02	11.98±0.18	4.79±0.02	9.71±0.02	4.32±0.06
20:1 (n-7)	0.66±0.02	0.24±0.01	0.68±0.02	0.26±0.00	0.58±0.02	0.22±0.01
22:1 (n-11)	12.56±0.05	5.04±0.04	13.82±0.03	5.85±0.08	10.73±0.06	5.24±0.09
22:1 (n-9)	1.37±0.00	0.42±0.01	1.46±0.07	0.49±0.00	1.25±0.00	0.30±0.00
24:1 (n-9)	0.69±0.28	0.09±0.03	0.89±0.02	0.12±0.00	0.75±0.01	0.08±0.01
SUM	**46.28±0.46**	**32.72±0.02**	**49.05±0.43**	**33.92±0.17**	**48.75±0.16**	**32.86±0.14**
16:2 (n-4)	0.29±0.03	0.32±0.00	0.32±0.02	0.39±0.00	0.25±0.00	0.34±0.00
16:3 (n-4)	0.54±0.09	0.49±0.01	0.58±0.03	0.48±0.00	0.45±0.05	0.47±0.01
16:4 (n-3)	0.44±0.12	0.45±0.01	0.48±0.09	0.55±0.00	0.34±0.06	0.49±0.01
18:2 (n-6)	4.46±0.06	11.39±0.01	3.81±0.00	9.14±0.06	6.61±0.04	12.17±0.07
18:2 (n-4)	0.40±0.07	0.13±0.01	0.23±0.01	0.15±0.00	0.29±0.03	0.14±0.00
18:3 (n-6)	0.13±0.05	0.35±0.00	0.09±0.00	0.24±0.00	0.11±0.05	0.27±0.00
18:3 (n-4)	0.81±0.07	0.10±0.00	0.73±0.01	0.11±0.00	2.11±0.06	0.10±0.00
18:3 (n-3)	1.47±0.06	1.97±0.01	1.54±0.02	1.78±0.05	1.30±0.05	2.13±0.01
18:4 (n-3)	0.09±0.02	1.48±0.00	0.08±0.00	1.73±0.01	0.08±0.02	1.54±0.02
20:2 (n-6)	0.27±0.01	0.28±0.00	0.23±0.01	0.29±0.01	0.24±0.01	0.26±0.01
20:3 (n-6)	0.40±0.01	0.40±0.00	0.25±0.00	0.26±0.00	0.31±0.02	0.31±0.00
20:4 (n-6)	3.91±0.02	4.41±0.01	2.39±0.03	2.72±0.08	3.06±0.00	3.18±0.01
20:3 (n-3)	0.09±0.01	0.14±0.01	0.09±0.00	0.15±0.00	0.08±0.00	0.14±0.01
20:4 (n-3)	0.36±0.00	0.45±0.00	0.37±0.01	0.51±0.01	0.31±0.00	0.47±0.01
20:5 (n-3)	6.19±0.01	6.11±0.03	6.54±0.09	7.06±0.01	5.60±0.01	6.35±0.03
21:5 (n-3)	0.26±0.01	0.31±0.07	0.25±0.00	0.42±0.01	0.22±0.01	0.35±0.03
22:5 (n-3)	0.91±0.06	0.91±0.01	0.86±0.00	0.99±0.00	0.77±0.01	0.91±0.00
22:6 (n-3)	9.35±0.02	10.49±0.05	9.08±0.09	10.43±0.03	8.51±0.04	10.13±0.03
SUM	**30.34±0.18**	**40.19±0.01**	**27.93±0.12**	**37.39±0.04**	**30.64±0.12**	**39.77±0.16**
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 n-3	19.15±0.01	22.31±0.01	19.29±0.12	23.61±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±0.05
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), $_1$: 1st batch of produced feed, $_2$: 2nd batch of produced feed

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.

	Diet 1		Diet 2		Diet 3		Wild
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	
n females	7	8	3	6	4	6	11
Fatty Acid							
14:0	3.40±0.39^{b}	3.28±0.26^{b}	3.61±0.14^{b}	3.44±0.24^{b}	3.38±0.31^{b}	3.17±0.23^{b}	2.02±0.21^{a}
15:0	0.26±0.02^{a}	0.28±0.02^{a}	0.25±0.00^{a}	0.28±0.02^{a}	0.24±0.04^{a}	0.27±0.02^{a}	0.29±0.05^{a}
16:0	16.65±0.74^{a}	16.99±0.84^{a}	17.41±0.87^{a}	17.04±0.42^{a}	16.65±0.66^{a}	16.85±0.67^{a}	17.15±0.75^{a}
17:0	0.26±0.06^{a}	0.28±0.05^{a}	0.25±0.05^{a}	0.29±0.05^{a}	0.31±0.04^{a}	0.24±0.03^{a}	0.60±0.10^{b}
18:0	6.66±2.18^{ab}	8.21±1.76^{ab}	7.34±3.09^{ab}	6.82±1.08^{ab}	6.05±0.97^{a}	9.10±1.39^{ab}	9.36±2.25^{b}
20:0	0.24±0.11^{a}	0.21±0.11^{a}	0.19±0.15^{ab}	0.18±0.11^{a}	0.23±0.03^{ab}	0.25±0.13^{ab}	0.38±0.02^{b}
SUM	**27.48±2.63^{a}**	**29.26±2.32^{a}**	**29.04±2.95^{a}**	**28.05±1.17^{a}**	**26.87±0.98^{a}**	**29.89±1.39^{a}**	**29.81±2.29^{a}**
14:1	0.13±0.01^{a}	0.13±0.01^{a}	0.13±0.01^{a}	0.14±0.01^{a}	0.13±0.02^{a}	0.12±0.01^{a}	0.42±0.06^{b}
16:1 (n-7)	7.94±0.26^{a}	7.66±0.26^{a}	8.21±0.25^{ab}	7.87±0.22^{a}	7.75±0.50^{a}	7.27±0.24^{a}	9.25±0.92^{b}
18:1 (n-9)	22.67±2.26^{a}	22.26±1.62^{a}	21.93±1.82^{abc}	22.40±0.98^{a}	25.72±1.09^{bc}	23.11±1.37^{ab}	27.69±1.50^{c}
18:1 (n-7)	2.82±0.34^{a}	2.54±0.25^{a}	2.78±0.36^{ab}	2.91±0.18^{a}	2.71±0.14^{a}	2.57±0.30^{a}	3.69±0.68^{b}
20:1 (n-9, n-11)	6.27±0.35^{c}	5.73±0.38^{b}	6.23±0.69^{bc}	6.42±0.41^{c}	5.25±0.45^{b}	5.37±0.36^{b}	1.45±0.13^{a}
20:1 (n-7)	0.35±0.02^{b}	0.34±0.03^{b}	0.35±0.02^{ab}	0.37±0.03^{b}	0.33±0.03^{ab}	0.32±0.04^{ab}	0.27±0.04^{a}
22:1 (n-11)	0.36±0.07^{ab}	0.47±0.47^{b}	0.38±0.09^{ab}	0.34±0.07^{ab}	0.27±0.06^{ab}	0.26±0.05^{ab}	0.05±0.01^{a}
22:1 (n-9)	0.14±0.03^{b}	0.14±0.01^{b}	0.15±0.06^{b}	0.16±0.02^{b}	0.13±0.01^{b}	0.13±0.01^{b}	0.05±0.01^{a}
24:1 (n-9)	0.25±0.02^{b}	0.27±0.03^{b}	0.27±0.01^{ab}	0.27±0.02^{b}	0.25±0.03^{b}	0.23±0.04^{b}	0.19±0.03^{a}
SUM	**40.93±2.46^{ab}**	**39.55±1.73^{a}**	**40.42±1.76^{ab}**	**40.87±0.66^{ab}**	**42.54±0.89^{ab}**	**39.38±1.76^{a}**	**43.06±1.97^{b}**
16:2 (n-4)	0.94±0.11^{ab}	0.88±0.08^{a}	0.96±0.06^{ab}	0.95±0.11^{ab}	1.00±0.13^{ab}	0.88±0.05^{a}	1.09±0.14^{b}
16:3 (n-4)	0.17±0.04^{a}	0.16±0.02^{a}	0.14±0.02^{a}	0.17±0.03^{a}	0.20±0.02^{a}	0.14±0.01^{a}	0.34±0.06^{b}
18:2 (n-6)	3.83±0.35^{c}	4.46±0.64^{d}	3.15±0.30^{abc}	3.54±0.24^{b}	4.33±0.40^{bcd}	4.62±0.49^{d}	1.62±0.32^{a}
18:2(n-4)	0.16±0.02^{a}	0.15±0.02^{a}	0.15±0.01^{a}	0.16±0.01^{a}	0.14±0.01^{a}	0.15±0.02^{a}	0.16±0.02^{a}
18:3 (n-6)	0.17±0.04^{a}	0.24±0.12^{a}	0.13±0.02^{a}	0.16±0.04^{a}	0.18±0.04^{a}	0.19±0.06^{a}	0.18±0.07^{a}
18:3 (n-4)	0.66±0.10^{a}	0.69±0.10^{a}	0.57±0.07^{a}	0.56±0.05^{a}	0.95±0.14^{b}	0.87±0.10^{ab}	1.78±0.17^{c}
18:3 (n-3)	0.17±0.04^{a}	0.19±0.05^{a}	0.17±0.02^{a}	0.16±0.02^{a}	0.17±0.02^{a}	0.16±0.05^{a}	0.17±0.02^{a}
18:4 (n-3)	0.19±0.01^{c}	0.17±0.02^{bc}	0.18±0.01^{bc}	0.18±0.01^{c}	0.17±0.02^{bc}	0.16±0.01^{b}	0.03±0.01^{a}
20:2 (n-6)	0.45±0.03^{b}	0.48±0.03^{b}	0.41±0.03^{ab}	0.49±0.04^{b}	0.48±0.01^{b}	0.58±0.06^{c}	0.37±0.05^{a}
20:3 (n-6)	0.50±0.04^{b}	0.52±0.07^{b}	0.47±0.01^{b}	0.49±0.07^{b}	0.46±0.02^{b}	0.55±0.05^{b}	0.30±0.04^{a}
20:4 (n-6)	2.93±0.43^{a}	3.32±0.21^{b}	2.10±0.17^{ab}	2.54±0.24^{a}	2.72±0.46^{ab}	2.80±0.15^{ab}	2.45±0.23^{a}
20:3 (n-3)	0.13±0.02^{a}	0.11±0.02^{a}	0.15±0.04^{a}	0.13±0.02^{a}	0.17±0.02^{a}	0.15±0.02^{a}	0.52±0.07^{b}
20:4 (n-3)	0.41±0.04^{b}	0.34±0.05^{ab}	0.42±0.02^{ab}	0.38±0.06^{ab}	0.33±0.06^{ab}	0.33±0.05^{ab}	0.33±0.05^{a}
20:5 (n-3)	3.28±0.27^{bc}	3.05±0.28^{bc}	3.56±0.30^{c}	3.24±0.35^{bc}	3.08±0.29^{bc}	2.87±0.26^{ab}	2.49±0.26^{a}
21:5 (n-3)	0.25±0.03^{b}	0.21±0.02^{b}	0.29±0.08^{b}	0.25±0.04^{b}	0.21±0.03^{b}	0.21±0.02^{b}	0.11±0.03^{a}
22:5 (n-3)	1.89±0.18^{b}	1.67±0.25^{ab}	1.92±0.15^{ab}	1.88±0.23^{b}	1.76±0.29^{ab}	1.66±0.09^{ab}	1.48±0.20^{a}
22:6 (n-3)	11.84±0.63^{b}	10.90±0.84^{b}	12.02±0.75^{b}	11.89±0.79^{b}	10.87±0.58^{b}	10.92±0.49^{b}	9.28±0.64^{a}
SUM	**27.95±1.21^{b}**	**27.55±0.56^{b}**	**26.78±0.60^{b}**	**27.17±0.98^{b}**	**27.23±0.91^{b}**	**27.23±1.20^{b}**	**22.70±0.96^{a}**
EPA:ARA	1.12±0.20^{bc}	0.92±0.12^{a}	1.70±0.05^{c}	1.28±0.13^{bc}	1.13±0.26^{abc}	1.02±0.10^{ab}	1.02±0.11^{ab}
DHA:EPA	3.60±0.26^{a}	3.57±0.30^{a}	3.38±0.48^{a}	3.67±0.56^{a}	3.53±0.31^{a}	3.80±0.30^{a}	3.73±0.45^{a}
SUM n-3	18.15±0.90^{d}	16.64±1.20^{bc}	18.69±0.66^{bcd}	18.12±0.82^{cd}	16.76±1.02^{bcd}	16.46±0.71^{b}	14.42±0.81^{a}
SUM n-6	7.89±0.34^{b}	9.02±0.89^{c}	6.26±0.22^{ab}	7.21±0.35^{b}	8.17±0.19^{bc}	8.73±0.44^{c}	4.91±0.61^{a}
n-6:n-3	0.43±0.02^{ab}	0.54±0.11^{d}	0.33±0.02^{abc}	0.40±0.02^{ab}	0.49±0.04^{bcd}	0.53±0.02^{cd}	0.34±0.05^{a}

Values represent average ± SD; Different lower-case letters represent a significant statistical difference (p = 0.05); ARA: arachidonic acid (20:4n-6); EPA: eicosapentaenoic acid (20:5n-3); DHA: docosahexaenoic acid (22:6n-3)

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.

	0 days post hatch			5 days post hatch		
	Diet 1		Wild	Diet 1		Wild
	Trial 1	Trial 2		Trial 1	Trial 2	
n females	4	4	6	3	3	5
Fatty Acid						
14:0	3.24±0.79^{b}	3.74±0.09^{b}	2.08±0.18^{a}	3.12±0.24^{B}	3.17±0.31^{B}	1.85±0.03^{A}
15:0	0.24±0.02^{a}	0.25±0.01^{a}	0.29±0.04^{a}	0.23±0.02^{A}	0.25±0.02^{A}	0.25±0.03^{A}
16:0	17.11±1.12^{a}	16.85±0.86^{a}	17.19±0.89^{a}	17.22±0.35^{A}	17.62±0.25^{A}	17.57±0.95^{A}
17:0	0.42±0.09^{a}	0.40±0.05^{a}	0.68±0.10^{b}	0.28±0.06^{A}	0.29±0.06^{A}	0.72±0.11^{B}
18:0	3.91±0.95^{a}	3.81±0.87^{a}	4.69±0.96^{a}	3.29±0.14^{A}	3.21±0.22^{A}	4.09±0.37^{B}
20:0	0.34±0.07^{a}	0.30±0.03^{a}	0.48±0.03^{b}	0.39±0.03^{A}	0.37±0.03^{A}	0.54±0.02^{B}
SUM	**25.25±1.48^{a}**	**25.35±1.01^{a}**	**25.40±1.66^{a}**	**24.53±0.21^{A}**	**24.90±0.04^{A}**	**25.04±1.18^{A}**
14:1	0.22±0.17^{a}	0.15±0.03^{a}	0.45±0.03^{b}	0.15±0.01^{A}	0.16±0.04^{A}	0.39±0.04^{B}
16:1 (n-7)	8.25±0.69^{a}	7.98±0.05^{a}	9.28±1.14^{a}	6.99±0.13^{A}	7.17±0.10^{AB}	8.18±0.78^{B}
18:1 (n-9)	27.20±3.38^{a}	25.58±0.99^{a}	32.83±1.68^{b}	24.85±0.46^{A}	24.47±0.55^{A}	31.01±1.84^{B}
18:1 (n-7)	3.70±0.23^{ab}	3.28±0.29^{a}	4.45±0.80^{b}	3.52±0.10^{A}	3.60±0.18^{A}	4.29±0.71^{A}
20:1 (n-9,n-11)	6.32±0.37^{b}	5.95±0.16^{b}	1.46±0.15^{a}	5.81±0.39^{B}	5.39±0.24^{B}	1.32±0.13^{A}
20:1 (n-7)	0.33±0.07^{a}	0.38±0.02^{a}	0.29±0.05^{a}	0.36±0.01^{B}	0.40±0.03^{B}	0.28±0.03^{A}
22:1 (n-11)	0.26±0.15^{b}	0.27±0.02^{b}	0.06±0.01^{a}	0.32±0.07^{B}	0.26±0.01^{B}	0.09±0.02^{A}
22:1 (n-9)	0.13±0.07^{ab}	0.16±0.01^{b}	0.07±0.04^{a}	0.20±0.02^{B}	0.21±0.05^{B}	0.07±0.05^{A}
24:1 (n-9)	0.00±0.00^{a}	0.00±0.01^{a}	0.04±0.08^{a}	0.31±0.01^{B}	0.32±0.07^{B}	0.00±0.00^{A}
SUM	**44.96±1.58^{a}**	**43.61±1.18^{a}**	**47.47±0.95^{b}**	**42.36±0.10^{A}**	**41.82±0.71^{A}**	**45.24±0.83^{B}**
16:2 (n-4)	1.14±0.20^{a}	0.97±0.11^{a}	1.34±0.08^{b}	1.21±0.02^{AB}	1.08±0.15^{A}	1.31±0.07^{B}
16:3 (n-4)	0.38±0.04^{a}	0.36±0.07^{a}	0.45±0.08^{a}	0.25±0.08^{A}	0.24±0.10^{A}	0.58±0.16^{B}
18:2 (n-6)	3.22±1.34^{b}	4.35±0.21^{c}	1.51±0.20^{a}	3.48±0.25^{B}	4.18±0.28^{C}	1.46±0.23^{A}
18:2(n-4)	0.14±0.04^{a}	0.22±0.04^{b}	0.08±0.02^{a}	0.16±0.03^{A}	0.27±0.05^{B}	0.10±0.04^{A}
18:3 (n-6)	0.13±0.05^{a}	0.17±0.01^{a}	0.19±0.10^{a}	0.16±0.01^{A}	0.17±0.01^{A}	0.17±0.09^{A}
18:3 (n-4)	0.87±0.40^{a}	0.68±0.06^{a}	1.73±0.16^{b}	0.57±0.05^{A}	0.60±0.05^{A}	1.55±0.18^{B}
18:3 (n-3)	0.15±0.04^{a}	0.18±0.04^{a}	0.16±0.01^{a}	0.13±0.01^{A}	0.16±0.04^{A}	0.16±0.02^{A}
18:4 (n-3)	0.13±0.08^{b}	0.17±0.02^{b}	0.04±0.02^{a}	0.14±0.02^{B}	0.14±0.02^{B}	0.04±0.01^{A}
20:2 (n-6)	0.43±0.06^{b}	0.45±0.03^{b}	0.36±0.02^{a}	0.44±0.03^{B}	0.43±0.04^{AB}	0.37±0.04^{A}
20:3 (n-6)	0.45±0.11^{b}	0.47±0.03^{b}	0.29±0.02^{a}	0.55±0.02^{C}	0.49±0.02^{B}	0.30±0.01^{A}
20:4 (n-6)	2.80±0.41^{ab}	3.15±0.30^{b}	2.39±0.19^{a}	3.58±0.34^{B}	4.04±0.36^{B}	2.89±0.18^{A}
20:3 (n-3)	0.23±0.12^{a}	0.15±0.03^{a}	0.48±0.05^{b}	0.16±0.04^{A}	0.12±0.05^{A}	0.49±0.08^{B}
20:4 (n-3)	0.39±0.06^{b}	0.30±0.02^{a}	0.31±0.02^{a}	0.38±0.01^{B}	0.27±0.02^{A}	0.31±0.03^{A}
20:5 (n-3)	3.09±0.42^{b}	3.16±0.25^{b}	2.36±0.15^{a}	3.22±0.19^{B}	3.40±0.28^{B}	2.52±0.21^{A}
21:5 (n-3)	0.18±0.06^{a}	0.23±0.05^{a}	0.13±0.08^{a}	0.24±0.02^{B}	0.21±0.02^{B}	0.13±0.05^{A}
22:5 (n-3)	1.72±0.43^{a}	1.65±0.11^{a}	1.35±0.13^{a}	1.86±0.23^{B}	1.65±0.01^{AB}	1.42±0.16^{A}
22:6 (n-3)	10.89±1.40^{b}	10.75±0.23^{b}	9.04±0.51^{a}	13.28±0.18^{B}	12.54±0.56^{B}	10.18±0.70^{A}
SUM	**26.34±3.60^{b}**	**27.42±0.81^{b}**	**22.22±0.90^{a}**	**29.79±0.15^{B}**	**29.97±0.94^{B}**	**23.98±1.18^{A}**
EPA:ARA	1.11±0.03^{a}	1.01±0.11^{a}	0.99±0.07^{a}	0.90±0.09^{A}	0.85±0.10^{A}	0.87±0.06^{A}
DHA:EPA	3.53±0.28^{a}	3.41±0.23^{a}	3.84±0.25^{a}	4.13±0.28^{A}	3.70±0.31^{A}	4.06±0.32^{A}
SUM n-3	16.79±2.27^{b}	16.60±0.36^{b}	13.86±0.51^{a}	19.40±0.09^{B}	18.48±0.63^{B}	15.25±0.71^{A}
SUM n-6	7.03±1.85^{b}	8.58±0.34^{b}	4.75±0.37^{a}	8.21±0.13^{B}	9.30±0.21^{C}	5.19±0.35^{A}
n-6:n-3	0.41±0.06^{b}	0.52±0.01^{c}	0.34±0.02^{a}	0.42±0.01^{B}	0.50±0.01^{C}	0.34±0.02^{A}

Values represent average ± SD; Different letters represent a significant statistical difference (p = 0.05; lower case at 0 and upper case at 5 days post hatch); ARA: arachidonic acid (20:4n-6); EPA: eicosapentaenoic acid (20:5n-3); DHA: docosahexaenoic acid (22:6n-3)

Paper II: Differential impacts of carp and salmon pituitary extracts on oogenesis, egg quality, molecular ontogeny and embryonic developmental competence in European eel

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Differential impacts of carp and salmon pituitary extracts on induced oogenesis, egg quality, molecular ontogeny and embryonic developmental competence in European eel

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Abstract

Low egg quality and embryonic survival are critical challenges in aquaculture, where assisted reproduction procedures among other may impact egg quality. This includes European eel, *Anguilla anguilla,* where pituitary extract from carp (CPE) or salmon (SPE) is applied to override a dopaminergic inhibition of the neuroendocrine system, preventing gonadotropin secretion and gonadal development. The present study used either CPE or SPE to induce vitellogenesis in female European eel and compared impacts on egg quality and offspring developmental competence with emphasis on the maternal-to-zygotic transition (MZT). Results showed that females treated with SPE produced significantly higher proportions of floating eggs with lower levels of cleavage abnormalities and higher embryonic survival. These findings related successful embryogenesis to higher abundance of mRNA transcripts throughout embryonic development of genes involved in cell adhesion, activation of MZT, and immune response (*dcbld1*, *epcam, oct4*, *igm*). In general, abundance of mRNA transcripts of *cldng*, *foxr1*, *cea*, *ccna1*, *ccnb1*, *ccnb2*, *zar1*, *oct4*, and *npm2* were relatively stable during the first eight hours, followed by a drop during MZT and low levels thereafter, indicating transfer and subsequent clearance of maternal mRNA. Moreover, mRNA abundance of *zar1*, *epcam,* and *dicer1* was associated with cleavage abnormalities, while mRNA abundance of *zar1*, *sox2*, *foxr1*, *cldn g*, *phb2*, *neurod4*, and *neurog1* (before MZT) was associated with later embryonic survival. In a second pattern, low initial mRNA abundance with an increase during MZT and higher levels persisting thereafter demonstrated the activation of zygotic transcription. Furthermore, mRNA abundance of *ccna1*, *npm2*, *oct4*, *neurod4*, and *neurog1* during later embryonic development was associated with hatch success. Together, the differences in offspring production and performance reported in this study indicate dissimilarities in PE composition impacting oogenesis and embryogenesis, in particular, the transition from initial maternal transcripts to zygotic transcription.

Introduction

High variability in egg quality and low survival during embryonic development pose a challenge to captive offspring production in fish aquaculture [1–4]. Here, embryonic and early larval developmental competence is influenced by intrinsic properties originating from the parents, as well as extrinsic factors, e.g. temperature experienced during incubation [2,5,6]. In relation to the former, many studies have focused on egg nutrient composition and effects of maternal nutrition, while the role of other cytoplasmic factors transferred to the developing oocyte, such as messenger RNAs (mRNAs) is less investigated [3,7]. The latter refers to maternal mRNA transcripts deposited into the egg during oocyte development, which are the essential drivers during zygotic and early embryonic development [5,7,8]. During the mid-blastula transition, the developmental control is taken over by the activation of the zygotic transcription [9,10]. Here, the successful process and timing of the maternal-to-zygotic transition (MZT), including the activation of zygotic transcription and the clearance of maternal mRNA, is essential for embryonic development [11–15]. Recent studies on various species have confirmed the tight relationship between the abundance of specific maternal mRNA transcripts, egg quality and embryonic developmental competence [16–21].

In female teleosts, vitellogenesis and follicular maturation are regulated by (1) two gonadotropins (GTH), follicle stimulating hormone (FSH) and luteinizing hormone (LH), (2) maturation-inducing steroid (MIS), and (3) maturation-promoting factor (MPF) [22]. Here, the production and release of FSH and LH is regulated by the brain and stimulate the synthetic agonists of gonadotropin-releasing hormone (GnRH). After being released into the bloodstream, LH and FSH target the ovaries regulating the production of sex steroids and oogenesis. While some species naturally enter sexual maturation and breed under suited captive conditions, the reproductive capability or other may be impeded in various ways. In such cases, artificial reproduction technologies may be efficient either to induce gametogenesis, trigger or synchronize spawning, or

enhance gamete quality and fertilization capacity [4]. For some species, manipulation of extrinsic factors, such as photoperiod and temperature, enable successful gametogenesis and reproduction, while in other cases, assisted reproductive protocols are applied depending on the species, the sex, and the state of the reproductive cycle that is targeted [4]. However, the hormonal manipulation controlling reproductive function may impact egg quality and offspring performance.

Species that do not reproduce naturally in captivity include anguillid eels. Being a high-value species in aquaculture, the development of hatchery technology is targeted. This is, however, hampered by their complex diadromous life cycle including long migrations to their spawning grounds. During the silvering process that marks the onset of migration, intrinsic inhibitory mechanisms at the brain pituitary level arrest puberty at an early stage [23]. This inhibition must be released when approaching the spawning area, but the endocrinological mechanisms remain unresolved. Consequently, efforts to develop breeding protocols must challenge the pubertal blockade that prevents gametogenesis and spawning. Here, hormonal treatments by application of exogenous gonadotropins have proven efficient in order to induce vitellogenesis and sustain oocyte development in breeding protocols [4,24]. In particular, pituitary extracts from carp (CPE) or salmon (SPE) have been applied as a source of LH and FSH, thereby inducing vitellogenesis, while follicular maturation is induced by 17α,20ß-dihydroxy-4-pregnen-3-one (DHP) [24,25].

For anguillid species, protocols using pituitary extracts have varied profoundly, both regarding product and dose. For instance, in the Japanese eel, *Anguilla japonica*, and in the European eel, *A. anguilla*, first attempts to induce maturation were conducted using CPE [26,27]. Later protocols have used either SPE e.g. *A. japonica* [28], *A. rostrata* [29], and *A. dieffenbachii* [30] or both PE types, as in *A. australis* [30,31] and *A. anguilla* [32]. In assisted reproduction studies of the latter species, CPE is applied in either constant [33–35] or increasing doses [36,37]. A recent study has compared two

treatment schemes for SPE and did not find an apparent effect of the hormonal treatment on progression of ovarian development [38]. Such artificial maturation protocols for European eel have led to a stable production of viable eggs up to the larval first-feeding stage [32,39,40]. Still, PE may vary in hormone composition, gonadotropins as well as other pituitary hormones, causing unintended variability in egg quality and survival during embryonic development, thereby affecting a stable, viable larval production. In this context, CPE and SPE may vary in their action through differences in accumulation of cytoplasmic components in the oocytes, such as mRNA during vitellogenesis, thereby affecting egg quality, acquisition of fertilization capacity and embryonic developmental competence.

We hypothesized that CPE and SPE may act differently due to differences in content and activity of GTH and other pituitary hormones and substances impacting early life history. Here, the embryonic development is largely unstudied in European eel, including the critical MZT, which takes place ~10 hpf at 18 °C. As such, this study examined experimentally differential impacts of CPE and SPE to induce oogenesis on spawning and offspring success, as well as molecular ontogeny. Unfertilized eggs and embryos were sampled throughout development for estimation of fertilization capability, occurrence of cleavage abnormalities, embryonic survival, and hatch success, as well as molecular analyses. Abundance of mRNA related to key genes involved in embryogenesis, such as cell cycle progression, cell division, cell adhesion, microRNA regulation, pluripotency regulation, cell signaling, activation of MZT, neurogenesis, and immune response were investigated throughout development and associated with developmental competence and hatch.

Material and Methods

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 approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696). Individual fish were anesthetized before tagging, biopsy, and stripping of gametes, and euthanized after stripping (females) or at the end of the experiment (males) using an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg L^{-1}, Sigma Aldrich, Germany).

Fish and experimental design

Broodstock establishment

For this study, female silver eels caught during down-stream migration were matured in two consecutive trials in 2016 and 2017. In 2016, 25 female eels (length = 76.18 ± 1.23 cm; weight = 879.16 ± 40.49 g) were caught at Lower Bann, Toomebridge, Northern Ireland, while in 2017, 26 female eels (length = 64.42 ± 1.21 cm; weight = 535.33 ± 39.93 g) were caught in Klitmøller Å, Lake Vandet, Denmark. The female eels were transported to the EEL-HATCH experimental facility in Hirtshals, Denmark, using an aerated freshwater tank. On arrival, fish were randomly distributed into replicated 1150 L tanks, connected to two Recirculating Aquaculture Systems (RAS), at a density of 10-15 females per tank, where one RAS unit was allocated for each hormonal treatment. In both years, fish were equally distributed into the two hormonal treatments. Overall, 27 females were allocated to CPE treatment (2016: n = 12; 2017: n = 15) and 24 females to SPE treatment (2016: n = 13; 2017: n = 11).

In both trials, male eels originated from Stensgård Eel Farm, where they were raised from glass eels on a formulated diet (DAN-EX 2848, BioMar A/S, Denmark) at a temperature of ~23 °C. In 2016, experiments comprised 60 male eels (length = 38.2 ± 2.1 cm, weight = 105.5 ± 15.3 g), and in 2017, 88 males (length = 38.5 ± 2.1 cm, weight = 114.7 ± 15.8 g). After transport to the facility, males were randomly distributed in four tanks (485 L) connected to a RAS unit at a density of ~15-20 eels per tank.

For acclimatization to oceanic conditions, salinity was gradually increased from 10 to 36 PSU over 14 days using Blue Treasure Aquaculture Salt (Qingdao Sea-Salt Aquarium Technology Co. Ltd. Qingdao, China), while temperature was adjusted from ~16 °C to 20 °C. Subsequently, each individual was tagged with a passive integrated transponder (PIT tag) in the dorsal muscle, while initial length and weight were recorded. During the experiment, male and female broodstock were maintained at ~20 °C and ~36 PSU under 12 h - 12 h light regime, with a 30 min twilight in the morning and evening to resemble the Sargasso Sea photoperiod.

Assisted reproduction and hormonal treatment

After acclimatization, vitellogenesis in the female broodstock was induced by weekly intramuscular injections of either CPE or SPE, each at 18.75 mg kg^{-1} initial BW for 10-21 weeks [41]. Salmon and carp pituitary extracts were obtained from Argent Chemical Laboratories, Washington, USA, diluted in NaCl 0.9 g/L, grinded, and centrifuged at 3600 RPM for 20 minutes, following [28,42] and supernatants stored at -20 °C until use. According to body-weight increase and oocyte developmental stage, monitored by biopsies, an additional injection of the respective hormone was given to each female as a primer [34,43]. After 12-24 hours the female received an injection of 17α,20ß-dihydroxy-4-pregnen-3-one (DHP) (Sigma-Aldrich, St. Louis, MO, USA) at 2 mg kg^{-1} current BW to induce follicular maturation and ovulation [42]. Males received weekly injections of

human chorionic gonadotropin (Sigma-Aldrich, Missouri, USA) at 150 IU/fish [41]. Prior to spawning, milt from 3-5 males was collected, sperm concentration standardized [44], and the dilution kept in an immobilizing medium [45]. Eggs were strip-spawned and fertilized using a standardized sperm to egg ratio [46,47]. After five min, eggs were transferred to 20 L buckets filled with ~15 L reverse osmosis water salted to ~36 PSU with Blue Treasure (Qingdao Sea-Salt Aquarium Technology Co., Ltd., Qingdao, China) at ~19°C. After 60 min, the floating layer of eggs/embryos was further transferred to a second bucket (as above) and kept for 60 min. Eggs/embryos were taken from the floating layer of the separation bucket and subsequently incubated in 10 1 L glass beakers (~5000 eggs/embryos per L) 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). Subsequent rearing occurred in a temperature incubator at 18°C [48] and 36 PSU. Additionally, 6 × 200 mL sterile tissue culture flasks filled with FUV seawater and supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) were stocked with eggs/embryos and incubated as above. Here, 3 flasks stocked with ~2500 eggs/embryos were used to follow embryonic development and 3 flasks stocked with ~600 eggs/embryos were used to analyze hatch success.

Data collection and image analyses

For each female, initial length and weight, weekly weights and weight at DHP injection were recorded as well as the number of weeks until spawning and the time between priming and DHP were recorded. Furthermore, the weight of stripped eggs (% initial weight) was recorded prior to fertilization and sampling of unfertilized eggs were conducted for determination of dry weight (3 × 0.1 mL) and gene expression (see below). For dry weight, samples were kept in the oven at 60°C for 24 h and weighed. At 0.5 hpf, the amount of floating eggs (%) was determined in a 25 mL volumetric

column. Digital images were used throughout the experiment to document oocyte, egg and embryonic development. Here, all images were taken with a Nikon Eclipse 55i microscope equipped with a Nikon digital sight DS-Fi1 Camera, while analyses used NIS Elements image software (Nikon Corporation, Tokyo, Japan). A digital image of the ovarian biopsy was taken, when females were primed or DHP was given and oil droplet size was measured subsequently. Here, ten of the largest oil droplets from ten oocytes at average stage were measured per female. In order to calculate fertilization success and embryonic survival, sub-samples from the embryonic development flasks were taken at 2, 3, 4, 5, 6, 7, 8, 16, 24, 32, 40, and 48 hpf and digital images were obtained. Fertilization success was measured from the digital images taken at 4 hpf, where eggs were categorized as fertilized when > 4 blastomeres could be observed and fertilization success was calculated as the percentage of fertilized divided by the total number eggs (obtained from the floating layer) [46]. At the remaining sampling points, embryonic survival was then calculated by counting the number of dead and alive eggs and expressing it as a percentage. Additionally, morphological measurements were conducted at 4 hpf, where total egg area, yolk area, and oil droplet area was measured. Cleavage abnormalities were also determined at 4 hpf by counting the number of eggs with regular and irregular cell cleavages. Hatch success from flasks was obtained by counting hatched larvae and dead eggs and was expressed as the number of hatched larvae divided by the total number of floating eggs.

Gene expression

Samples for gene expression were taken from the ovarian tissue of the female after spawning, the unfertilized eggs and embryos at 2, 4, 8, 24, 32, and 48 hpf and stored in Eppendorf vials filled with RNA later, kept in the fridge at 4°C for 24 hours and at -20°C until analysis. RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Germany) according to manufacturer's

instructions. RNA concentration and purity was analyzed by spectrophotometry using Nanodrop One (Thermo Fisher Scientific, USA). From the resulting total RNA, 1 µg was transcribed using qScript cDNA Synthesis Kit (Quantobio, Germany), following the manufacturer's instructions. This included a step to permanently inactivate all trace levels of DNase activity using PerfeCta DNase I (RNase free) (Quantabio, Germany). Primers of 10 genes were retrieved from previous studies (Table1). Primers of 14 further genes were designed using primer 3 software v 0.4.01 based on the coding sequence (cds), which were retrieved based on the predicted annotation of the European eel reference genome [49] or with the available cds of *Anguilla japonica* when the annotation was not available. All primers have been tested in silico for specificity using blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, from all samples, the expression of 24 genes (Table1) was analyzed with two technical replicates using the qPCR BiomarkTM HD system (Fluidigm) based on 96.96 dynamic arrays (GE plates), as previously described [50]. In brief, a pre-amplification step was conducted with a 500 nM pool of all primers in PreAmp Master Mix (Fluidigm) and 1.25 µL cDNA per sample run in a thermocycler for 2 min at 95°C; 10 cycles: 15 s each at 95°C and 4 min at 60°C. Obtained PCR products were then diluted 1:5 with low EDTA-TE buffer. The preamplified product was loaded onto the chip with SsoFast-EvaGreen Supermix Low Rox (Bio-Rad) and DNA-Binding Dye Sample Loading Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 µM in Assay Loading Reagent (Fluidigm) and low EDTA-TE Buffer. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60°C. qBase + software verified stability of housekeeping gene expression throughout analyzed samples ($M < 0.4$; according to [51]). Gene expression was normalized (ΔCt) to the geometric mean of the four most stable reference genes (*ccna2*, *cei*, *thaa*, *igfr-1b*). Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method, in relation to a random unfertilized egg sample, according to [52].

Statistical analyses

Data were analyzed 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 [56]. The effect of hormonal treatment on embryonic survival throughout development (2, 3, 4, 5, 6, 7, 8, 16, 24, 32, 40, 48 hpf) and on gene expression throughout development (unfertilized egg, 2, 4, 8, 24, 32, 49 hpf) were tested using a series of repeated measures mixed-effect model ANOVAs. 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 [57]. The effect of hormonal treatment on initial length, initial weight, time until spawning, oil droplet stage at priming and at DHP stage, weight increase of females, stripped eggs, floating eggs, dry-weight of unfertilized eggs, fertilization success, cleavage abnormalities and hatch success were tested using student's t-tests. Moreover, a linear and quadratic regression function were used to analyze the relationship between cleavage abnormalities at 4 hpf and embryonic survival at 48 hpf, as well as relationships between gene expression in the ovary and unfertilized eggs as well as between gene expression and offspring quality parameters. In case both regression functions were significant, F-statistics were used to evaluate best fit.

For female and quality parameters, ANOVA models were first run testing the effect of broodstock series (2016, 2017), hormonal treatment and their interaction. While initial length ($p < 0.0001$) and weight ($p < 0.0001$) differed between female broodstock from the two locations, quality

parameters were similar among female broodstock, including fertilization success ($p = 0.7832$), cleavage abnormalities ($p = 0.6711$), embryonic survival ($p = 0.4538$), and hatch success ($p = 0.6258$). Therefore, data from the two experiments were pooled. Comparing the two resulting treatment groups, no significant difference was found in the initial weight between CPE (686. 3 ± 49.7 g) and SPE females (723.5 ± 55.9 g; $p = 0.620$). Likewise, the initial length between females from CPE (70.1 ± 1.6 cm) and SPE (70.3 ± 1.8 cm) treatment did not differ ($p = 0.952$).

Results

Reproductive success and offspring development

Overall, 51 females were included in the combined analyses of the two trials. Female information and reproductive success for each treatment are shown in Table 2. The weight increase (% IW) until DHP stage was similar for the two treatments ($p = 0.161$). Also, the number of weeks until spawning ($p = 0.189$) and the time between primer and DHP ($p = 0.659$) did not differ between hormonal treatments. Moreover, there was no difference between hormonal treatments in the oil droplet size at priming stage ($p = 0.142$), neither at DHP stage ($p = 0.139$). The overall percentage of females that matured was higher in the CPE treatment, while the percentage of stripped females producing fertilized eggs and hatched larvae was higher in the SPE treatment (Table 2).

The amounts of stripped eggs relative to body weight ($p = 0.926$) and the dry-weight of unfertilized eggs ($p = 0.865$) did not differ between CPE and SPE females. While the percentage of floating eggs was higher in females from the SPE treatment than in females from the CPE treatment ($p = 0.038$), the fertilization success of eggs from the floating layer ($p = 0.868$) and egg size at 4 hpf ($p = 0.428$) and did not differ. In contrast, embryonic survival was higher for SPE treated females than CPE ($p = 0.029$; Fig 1A), while embryonic survival in general decreased over time ($p = 0.003$;

Fig 1B). This coincided with higher proportions of cleavage abnormalities at 4 hpf in embryos obtained from CPE treated females ($p = 0.037$; Fig 1C). Here, the relationship between cleavage abnormalities and embryonic survival at 48 hpf was significant for both treatments (Fig 1D, E). An example or normal egg development and the occurrence of cleavage abnormalities is shown in Fig 2.

The number of females with viable offspring decreased over time to about half of the CPE and three quarters of the SPE females succeeding in larval production (Table 2). The resulting hatch success was on average higher for SPE (37.87 ± 7.25 %) than CPE (26.54 ± 6.19 %), but the difference between treatments was not significant ($p = 0.245$).

mRNA transcript abundance and gene expression patterns

Detailed results of gene expression analyses are shown in Table 3. Here, the relationship between expression levels in the ovary and the unfertilized egg for SPE was significant for 13 genes (*cdhr2*, *dcbld1*, *epcam, foxr1*, *cea*, *ccna1*, *ccnb1*, *oct4*, *sox2*, *neurod4*, *c3*, *igm*, *il1β*). For CPE, the relationship was significant for 7 genes (*cdhr2*, *epcam*, *ccna1*, *oct4*, *neurog1*, *c3*, *igm*) with six of these overlapping between treatments. For all genes, the relationship was best described by a positive linear regression.

The statistical model for analysis of hormonal treatment and age on mRNA abundance from unfertilized eggs throughout embryogenesis showed that the transcript abundance of eight genes differed significantly (Table 3). Here, *dcbld1*, *epcam*, *oct4*, and *igm*, which are associated with cell adhesion, MZT activation, and immune response, showed significantly higher mRNA abundance in offspring from SPE than CPE treated females. In contrast, *cdhr2*, *cldng*, *foxr1*, and *ccnb1*, associated with cell division, cell cycle control, and cell adhesion, showed significantly higher mRNA levels in offspring from CPE than SPE treated females. Moreover, significant variations in patterns of mRNA

abundance throughout embryonic development were observed for all genes, with the exception of *cdhr2* and *dcbld1*. Here, *cldng*, *foxr1*, *cea*, *ccna1*, *ccnb1*, *ccnb2*, *zar1*, *oct4*, and *npm2* showed a similar pattern with relatively stable abundance during the first eight hours until MZT (Fig 3A, B). Hereafter, a strong decline in mRNA abundance was observed for these genes, remaining low until hatch. On the other hand, mRNA abundance of *sox2*, *neurod4*, *neurog1*, *phb2*, and *c3* was low in the unfertilized eggs and first hours post fertilization, but increased steeply during MZT and remained high thereafter (Fig 3C, D). Up to almost 100,000-fold differences were detected in the expression during later embryonic development. Lastly, abundance of mRNA transcripts were low for *epcam*, *dicer1*, *igm*, *il1β,* while a deviating pattern with large differences between treatments was observed for *dcbld1* during embryonic development (Fig 3E, F).

mRNA abundance and developmental competence

The relationships between relative mRNA abundance of analyzed genes and offspring quality parameters are shown in Table 4. Significant relationships between relative mRNA abundance at 2 hpf and the occurrence of cleavage abnormalities (at 4 hpf) were found for three genes. For *dicer1*, no relationship was found for CPE females, however, for SPE females the relationship was best explained by a negative linear regression. The relationship between *epcam* and cleavage abnormalities for CPE females was best explained by a negative linear regression, while no relationship was found for the SPE treatment. The relationship for *zar1* was best explained by a negative linear regression for SPE females, but was not significant for CPE females.

For seven genes, the relationship between the relative mRNA abundance at 8 hpf and embryonic survival at 48 hpf was significant for at least one of the treatments. For *zar1*, relationships were best explained by positive linear regressions for CPE as well as SPE. The relationship between *sox2* and survival was also best explained by positive linear regressions for both treatments. On the contrary,

no significant relationship was found between the mRNA abundance of *foxr1* and survival for CPE females, while a significant positive linear regression was found for SPE females. The relationship between the mRNA abundance of *cldng* and survival was also non-significant for CPE females but best explained by a positive linear regression for the SPE treatment. Similarly, no significant relationship was found for CPE females between *phb2* abundance and survival, while the relationship for SPE females was best explained by a positive linear regression. For *neurod4*, a significant relationship was found for both treatments. Here, the relationship for CPE females was best explained by a positive linear regression, whereas for SPE females it was a positive quadratic regression. Similar results were found for *neurog1*, where the relationship for CPE females was best explained by a positive linear and for SPE females by a quadratic regression.

Lastly, five genes showed a significant relationship between the mRNA abundance at 32 hpf and hatch success. The relationship between the mRNA abundance of *ccna1* and hatch success was best explained by a positive quadratic regression for CPE and a positive linear regression for SPE females. For *npm2*, no relationship was found for CPE females, while the relationship was best explained by a positive linear regression for SPE females. For *oct4*, significant relationships were found for both treatments and best explained by a positive quadratic regression for CPE and a linear regression for SPE females. No significant relationship was found between the abundance of *neurod4* and hatch success for CPE females, however, for SPE females, the relationship was best explained by a negative quadratic regression. Similarly, no relationship was found between the abundance of *neurog1* and hatch success for CPE females. Here, the relationship for SPE females was best explained by a negative linear regression.

Discussion

This study identified the differential impacts of carp and salmon pituitary extract administration on ovarian development, egg quality and embryonic developmental competence in European eel. This included the mRNA abundance of 20 genes involved in zygotic formation and embryogenesis. Here, several of these were associated with embryonic survival, occurrence of cleavage abnormalities, and/or hatch success.

Assisted reproduction

Assisted reproduction techniques are commonly used in aquaculture to enhance reproductive success including a variety of hormonal treatments [4]. Treatment protocols used for induction of vitellogenesis in anguillid eels primarily apply extracts derived from mature carp or salmon pituitaries as therapeutic agents, overriding the dopaminergic inhibition of gonadotropins. In the present study, both CPE and SPE induced vitellogenesis and led to production of viable larvae, however, with differences in responsiveness and embryonic survival. Here, more CPE females reached the follicular maturation stage, while the number of SPE females with fertilized eggs and hatched larvae was higher. Such discrepancy could be caused by differences in active pituitary hormones, particularly FSH and LH, affecting follicular and ovarian development. In salmonids, which show synchronous development of oocytes, FSH gene expression is predominant during the early stages of oogenesis, whereas LH gene expression becomes elevated at the late stages of oogenesis. In contrast, in multiple spawners such as sea bream, *Sparus aurata*, goldfish, *Carassius auratus*, and gourami, Osphronemidae, which show asynchronous development of oocytes, the expression of both genes increases with the progression of ovarian maturity and peaks during the spawning season [58–60]. The treatment, and in this case, differences in active hormonal substances pituitary extracts, including FSH and LH, may have a high impact on egg quality making eel an interesting model species to

compare treatments. In Japanese eel, sequential expression of FSH and LH at the brain pituitary level has been documented throughout induced ovarian development [58,61] and a similar pattern was also found in European eel with increasing levels of LH and decreasing FSH levels throughout experimental maturation [62]. In comparison, commercially available pituitaries glands originate from mature fishes with a relatively constant hormone composition, thus the match with the species from which the pituitaries originate as well as their reproductive developmental stage will influence the effectiveness of treatment. Differences in response to hormonal therapy may be more pronounced in anguillid eels than in most other fish species, as they require repeated treatment, most commonly weekly, for a prolonged period to sustain vitellogenesis and reach follicular maturation.

During oogenesis mRNA transcripts as well as nutrients are incorporated into oocytes during follicular development [5]. Maternal mRNAs and proteins, which are loaded into the egg during oogenesis, implement basic biosynthetic processes in the early embryo with subsequent clearance during MZT [7,10]. We found significant relationships between mRNA abundance in unfertilized eggs and the remaining cohorts of oocytes in ovary after stripping, with significant differences between SPE and CPE treated females for genes related to cell division, cell cycle control, cell adhesion, MZT activation, neurogenesis, and immune response. The abundance of a number of these mRNA transcripts also differed between treatment during embryogenesis which may explain the higher amounts of floating eggs, higher embryonic survival, and lower frequency of cleavage abnormalities in offspring from SPE treated females. The occurrence of cleavage abnormalities has been related to embryonic mortality in various species, such as Atlantic cod, *Gadus morhua* [63], yellowtail flounder, *Limanda ferruginea* [64], turbot, *Scophthalmus maximus* [65]. In this study, the occurrence of cleavage abnormalities led to high embryonic mortality. Furthermore, the cleavage abnormalities clearly connected the observed lack of cell adhesion with differences in gene expression patterns.

Cell adhesion

Abundance of mRNA transcripts of two genes related to cell adhesion differed among treatments, In particular, SPE treatment was positively associated with higher mRNA abundance of *dcbld1* and *epcam*. Expression of *dcbld1* is associated with cell adhesion during embryonic development, yet, its role is still unknown [66]. In contrast to the present study, Rise et al. [67] found elevated *dcbld1* abundance in the lowest quality eggs compared to the highest quality ones in Atlantic cod. However, variation was high with larger than 100-fold differences among females. When including all females in the analysis, no relationship between *dcbld1* abundance and egg quality was found. In our study, *dcbld1* abundance also showed considerable variation between batches. Moreover, the relation between unfertilized egg and ovary indicated a strong maternal effect. Interestingly, higher mRNA abundance in SPE compared to CPE females may relate to incomplete cell adhesion observed and compromising development in European eel embryos.

The other gene, *epcam* affects cell adhesion, migration, proliferation, differentiation and signaling [68–70]. Possible influence in fish embryogenesis has been associated with high abundance in early stages of Atlantic cod [71] and zebrafish, *Danio rerio* [72]. Thus, in zebrafish, expression of *epcam* is required for epithelial morphogenesis during epiboly [68] as well as cell migration in the lateral line system [73]. In our study, high mRNA abundance manifested maternal transfer of *epcam* to eel eggs and embryos with a positive effect of treatment for embryos obtained from SPE females. Moreover, a negative relationship with cleavage abnormalities was found for CPE. Together, these results propose that high abundance of this gene also is important for normal development in European eel embryos.

MZT activation

The expression patterns of *oct4* have been studied in medaka, *Oryzias latipes* [74–77], Nile tilapia, *Oreochromis niloticus* [78,79], and zebrafish [80–84] documenting that it is essential for normal fish embryonic development and survival. In zebrafish, maternally inherited *oct4* together with *sox2* (*soxb1*) and *nanog*, are responsible for activating the zygotic expression and initiating the clearance of maternal mRNA through activation of microRNA, hence it is considered fundamental for successful MZT [82]. In accordance, we found high mRNA abundance of *oct4* in ovaries, eggs, and embryos, with highest abundance obtained from SPE treatment, suggesting that maternally inherited *oct4* similarly plays an important role in successful MZT and survival during embryonic development in eel. Furthermore, abundance of *oct4* at later embryonic stage were linked to higher hatch rates in both treatments, which indicate that *oct4* has an additional role during later embryonic development, important for successful hatch.

Immune response

During early development, teleost fish rely exclusively on the innate arm of the immune system until their adaptive immune system is sufficiently developed [85]. The maternal transfer of immune-related factors to the eggs has been proposed for several species and is likely involved in early protection of the embryo, however, the exact mechanisms are still unknown [86–88]. Among published studies, maternally transferred *igm* has been associated with higher larval survival in sea bream [89] and Indian major carp, *Labeo rohita* [90]. Moreover, the maternal antibody transfer improved the protection against pathogenic attacks for developing embryos of zebrafish [91]. In European eel, the molecular ontogeny of the immune system has been studied for larval stages from hatch until first-feeding at different temperatures [55], identifying an array of candidate genes

involved in early immune response, several of which were also investigated in this study. Present results showed the maternal transfer of *igm* mRNA in European eel to the eggs, potentially having an important role during embryonic development. Thus, higher mRNA levels in embryos obtained from SPE treated females might indicate a strengthened immune-readiness due to maternally derived immune factors. While our results showed maternal transfer of *c3* and *il1β* transcripts, their presence throughout embryonic development followed different patterns used also to categorize the other genes.

mRNA transcript profiles and MZT

Overall, the mRNA abundance of 20 genes revealed differential expression patterns throughout development with three main patterns. In the first pattern, high abundance in early embryonic development (until 8 hpf) was observed, with a subsequent drop in mRNA levels after the MZT (between 8 and 24 hpf), likely demonstrating the transfer and subsequent clearance of maternal mRNA. This pattern was found for *cldn g*, *foxr1*, *cea*, *ccna1*, *ccnb1*, *ccnb2*, *zar1*, *oct4*, *npm2*, which all are genes involved in early developmental functions, such as cell adhesion, cell division, cell cycle control, oocyte-embryo transition, and MZT activation. This pattern compares to maternal-effect genes and is known from other species for genes with these functions [67,92–94]. In the second pattern, low mRNA levels during the early embryonic stages (until 8 hpf) were observed, with an increase after 8 to 24 hpf, probably demonstrating the activation of zygotic transcription. This was the case for *sox2*, *neurod4*, *neurog1*, *phb2*, and *c3*, primarily genes involved in neurogenesis but also in cell signaling and immune response. This pattern of starting transcription during MZT is common for genes involved in processes such as organogenesis [93]. In a third pattern, less drastic changes in transcription throughout development were observed for *cdhr2*, *epcam*, *dicer1*, *igm*, *il1β*, genes involved in cell adhesion, microRNA regulation and immune response. Interestingly, a deviating

pattern between treatments was observed for *dcbld1*, which appeared to follow the maternal-effect gene pattern only for embryos obtained from SPE treated females.

Genes of pattern one included c*ldn g*, a member of the family of claudins, which are known for their important function generating tight junctions between cells in teleosts [95–97]. C*ldn g* appears to be highly abundant during early embryonic development in zebrafish [72], which was also the case in the present study with highest levels for CPE, however, its exact function has not yet been described for European eel. Ovarian-specific expression of *foxr1* has been shown in zebrafish [98], freshwater medaka, *Oryzias melastigma* [99], rice field eel *Monopterus albus* [100], and European eel [54] indicating a possible important role during early life history. Recently, vital importance of *foxr1* transcript abundance on embryonic survival around MZT was suggested for zebrafish [98], which is consistent with high our results. Also, the family of cyclins is essential for early cell cycle progression in teleosts and is highly expressed in early embryonic stages [72,92,101,102]. In rainbow trout, abundance of *ccna1* might be linked to embryonic developmental competence [103]. In this study, mRNA abundance was not associated to embryonic survival, yet, gene expression levels at later stages were related to hatch success, which may indicate an additional function during later embryonic development in European eel. mRNA abundance patterns of *zar1*, first shown to be critical for the oocyte-to-embryo transition in mice [104], suggest an important role in early development of rainbow trout, *Oncorhynchus mykiss* [103,105] and Atlantic cod [92].

Sox2, relating to the second pattern has been shown to be responsible for the successful activation of the MZT in zebrafish, together with *oct4* and *nanog* [82]. The expression patterns of *sox2* in our study are in accordance with this, indicating a similar function in European eel. For *phb2,* a previous study on European eel found that the abundance of transcripts was higher in a “high hatch group” compared to a “low hatch group” [18]. Our similar results further advocate an important role

of this gene during embryonic development in European eel. In contrast, mRNA abundance of this gene in rainbow trout has been negatively correlated with developmental success indicating species specific differences [106]. Another important process during embryonic development is neurogenesis, which in teleosts mainly has been studied in zebrafish, where *neurod4* and *neurog1* are key players [107–110]. In this study, their mRNA abundance patterns suggested importance already during early development. Interestingly, a negative relationship between their mRNA abundance during late embryonic development and hatch success was found, which calls for more detailed research on the function of these two genes during eel embryogenesis.

Representing the third pattern, *dicer1* has been ascribed an essential role in microRNA (miRNA) synthesis during embryonic development. It has mainly been investigated in zebrafish [8,111], where lack of *dicer1* led to slower growth rates and shorter survival [112], as well as abnormal persistence of maternal mRNA beyond MZT [11,113]. Moreover, coinciding with our results, a high abundance in rainbow trout embryos suggest an important role during early development [114]. In addition to the high abundance and importance before MZT, an increase in the expression towards larval hatch was found in our study, indicating a further possible important role during the larval stages in eel.

In particular, the association between mRNA abundance at 2 hpf of three genes, *zar1*, *epcam*, *dicer1* with cleavage abnormalities as well as mRNA abundance before MZT (8 hpf) of seven genes, *zar1*, *sox2*, *foxr1*, *cldn g*, *phb2*, *neurod4*, *neurog1* with later embryonic survival (48 hpf) indicated maternal mRNA transfer and their importance for successful development in European eel embryos. Moreover, the expression of five genes, *ccna1*, *npm2*, *oct4*, *neurod4*, *neurog1* during later embryonic development (32 hpf) associated with hatch success, emphasized the importance of their transcription during the transition from maternal mRNA control to zygotic transcription. Thus, assisted

reproduction protocols have the potential to generate high quality eggs and embryos, however, these processes, which are vital for successful later embryonic development and hatch of viable larvae, need further attention.

Together, our results showed that viable larval production of European eel at a commercial scale can be established using assisted reproduction, while oogenesis and offspring developmental competence was influenced by the therapeutic agent applied. Such differences are likely due to variable levels of active pituitary hormone in extracts, particularly FSH and LH. Our study suggest higher levels of FSH in CPE and lower LH, while SPE may have higher LH but lower FSH levels. On the one hand, higher FSH levels would explain higher responsiveness of CPE treated females, and is supported by the often applied CPE protocol using an increasing dose [36,37]. On the other hand, higher LH levels in SPE would explain higher success rates during follicular maturation and spawning. Further research is needed to clarify the physiological requirements of gonadotropins and the progression of LH and FSH during the oogenesis. Here, development of ELISA would contribute to precise measurements of LH and FSH levels in pituitary extracts to get further information on the quality [115,116]. This information together with potential influences of other pituitary hormones might lead to development of alternative therapeutics for example employing recombinant hormones adapted to the batch spawning reproductive strategy of eel [30,43]. Here, recent advances in recombinant hormone technology that have been successfully confirmed for Japanese eel, may be beneficial [115]. Establishing commercial captive hatchery production of eel would benefit aquaculture production as well as conservation measures for endangered eel species.

Conclusion

This study represents a first step in elucidating the complex mechanisms underlying embryogenesis in European eel including maternal mRNA transfer to offspring and subsequent activation of zygotic transcription. The analyses revealed critical genes for normal embryonic development, consistent with findings in other fish including species of aquaculture relevance. Although, both CPE and SPE successfully induced oogenesis, influences on egg quality and embryonic developmental competence differed with higher embryonic survival success of SPE treated females. Complementing mRNA transcript analyses showed that embryonic survival was related to differential expression in eight genes involved in cell adhesion, cell division, cell cycle control, MZT activation, and immune regulation. Differences in response to treatment suggest that dissimilarities in PE composition of FSH and LH affect the incorporation of mRNA into the egg thereby influencing the embryos developmental competence. Overall, differential expression patterns throughout embryonic development were observed for 20 genes involved in key mechanisms during early development showing either increasing or decreasing expression profiles around the MZT. Here, maternal mRNA as well as the successful transition from maternal transcripts to zygotic transcription appeared to be of high importance for healthy embryonic development. Future studies including transcriptomics would be relevant in order to substantiate knowledge on incorporation of mRNA transcripts during ovarian development as well as the importance of transcript abundance of different genes during eel embryogenesis.

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Table 1. Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR. Full name and abbreviation is given for each gene with function, accession numbers and references for primers retrieved from previous studies. Primers that were designed based on the coding sequence (cds), that have been retrieved based on the predicted annotation of the European eel reference genome [49] are indicated with the "-" symbol, complemented with cds of *A. japonica*.

Abbreviation	Full name	Function	Primer Sequence (5' 3') (F: Forward; R: Reverse)	Reference/Accession
ccna2	Cyclin A2	Reference	F: ATGGAGATAAAATGCAGGCCT	AB061443.1
			R: AGCTTGCCTCTCAGAACAGA	
cei	Cellular island	Reference	F: CCTCAAACACCCCAACATCC	-
			R: AGCTCCTCCATGTACGTTGC	
thαa	Thyroid hormone receptor *αa*	Reference	F: GCAGTTCAACCTGGACGACT	Politis et al., 2018
			R: CCTGGCACTTCTCGATCTTC	
igfr-1b	Insulin like growth factor receptor 1b	Reference	F: ATGGGAATCTTCAGCTCTTTAGA	-
			R: TCAAACTCCTCCTCCAAGCT	
foxr1	Forkhead box R1	Cell division	F: CCTCGTCCAGCGAATATCTTCTT	Geffroy et al., 2016
			R: TGTTTTGAGCGAGATTCAGCTTC	
cea	Cellular atoll	Cell division	F: AGCACTCTGTCGAAGGAAGT	-
			R: ACCTTGATCTTCCCCACCAG	
ccna1	Cyclin A1	Cell cycle control	F: ACCTGCTTCTCAAGGTCCTC	AB061442.1
			R: CCTTGGACGGAACATGTAGC	
ccnb1	Cyclin B1	Cell cycle control	F: TCAACCTCAAGCTGACGGAG	AB183431.1
			R: CTGCATCTCCCACACCCAT	
ccnb2	Cyclin B2	Cell cycle control	F: GTGTTGCATGATGGGCTTGA	AB183432.1
			R: TGATGCAGAGAAACACACGC	
npm2	Nucleoplasmin 2	Cell cycle control	F: AAAGTTGACCGTTGGACCAG	Rozenfeld et al., 2016
			R: GGCCTATGTGAGGCAGTCAT	
cdhr2	cadherin-related family member 2 or protocadherin-24	Cell adhesion	F: GTTCCTTCGGTCACCACAAC	-
			R: TGTGTGACCAGGTGCAAATG	
cldn g	Claudin g	Cell adhesion	F: CTCCCCAGCCAATGAACAAC	-
			R: ATTCTGTTGTCGGTTGCTGG	

dcbld1	Discoidin, CUB and LCCL domain containing 1	Cell adhesion	F: ACCAGTCCACAGAGTTCACC	-
			R: CGTGTGCAGGTAGTCGTAGT	
epcam	Epithelial cell adhesion molecule	Cell adhesion	F: TCTTCAGGTCTCTCTCGATGT	-
			R: GCTGGTGAAGGAATATACTCTGG	
zar1	Zygote arrest 1	Oocyte-embryo transition	F: TGAGGTTTCAGTTCTTGGAGCAG	Geffroy et al., 2016
			R: TAAACCTTGTTGGTTCCCTGGAC	
dicer1	*Dicer1*	microRNA regulation	F: CGGTCGTCTTAAACAGGCTTATA	-
			R: ACCTCCTCCTGTTTGCGAAA	
oct4 (also known as *pou5f1* or *pou2*)	Octamer-binding transcription factor 4	Pluripotency regulation/ MZT activation	F: AACAGTTTGCCAAGGAGCTG	-
			R: GCACATGTTCTTAAAGCTCAGC	
sox2	Sox2	Pluripotency regulation/ MZT activation	F: GTCCTTTCATCGACGAAGCG	-
			R: TGATTTACTCCCGCACCCAA	
phb2	Prohibitin 2	cell signaling	F: AAATGTTGGGAGAGGCTGTG	Rozenfeld et al., 2016
			R: ACCGTCTTGGCGATATTCTG	
neurod4	Neuronal differentiation 4	Neurogenesis	F: TTCCTGTCCTCGCACCAGTA	Politis et al. (unpublished data)
			R: AAGGAGTCGAAGGCCATGTC	
neurog1	Neurogenin 1	Neurogenesis	F: CAGGATGCACAACCTCAATG	Politis et al. (unpublished data)
			R: TGCAATTCGGATTGTCTCTG	
c3	Complement component c3	Immune response	F: AATATGTGCTCCCAGCCTTC	Miest et al., 2019
			R: GATAACTTGCCGTGATGTCG	
igm	Immunoglobulin M	Immune response	F: CCAAGGACCATTCTTTCGTC	Miest et al., 2019
			R: ACTGGCTTTCAGGAAGATGC	
il1β	Interleukin 1β	Immune response	F: ATTGGCTGGACTTGTGTTCC	Miest et al., 2019
			R: CATGTGCATTAAAGCTGACCTG	

Table 2. Data on females and reproductive success of European eel, *Anguilla anguilla.* Different lower-case letters represent a significant statistical difference ($p < 0.05$).

Parameter	**CPE**	**SPE**
n females in trials	27	24
Time until spawning (wks)	14.19±3.93 [a]	15.58±2.61 [a]
Time between primer and DHP (h)	20.38±0.75 [a]	21.24±1.05 [a]
Oil droplet diameter at priming stage (µm)	109.9±33.68 [a]	95.33±26.94 [a]
Oil droplet diameter at DHP stage (µm)	157.4±31.9 [a]	145.1±16.84 [a]
Stripped females (%)	96.3 (n = 26)	79.2 (n = 19)
Stripped females with fertilized eggs (%)	57.7 (n = 15)	89.5 (n = 17)
Stripped females with hatched larvae (%)	50.0 (n = 13)	73.7 (n = 14)
Weight increase (% IW)	22.9±1.9 [a]	26.0±0.9 [a]
Stripped eggs (% IW)	42.4±1.9 [a]	42.1±2.4 [a]
Floating eggs (%)	55.3±8.8 [a]	79.7±6.1 [b]
Dry-weight unfertilized egg (mg egg^{-1})	0.060±0.002 [a]	0.061±0.001 [a]
Egg size (mm^2)	1.58±0.09 [a]	1.68±0.09 [a]
Fertilization success (%)	53.37±4.95 [a]	54.75±6.56 [a]

Table 3. **Gene expression in European eel, *Anguilla anguilla.*** Best described relationship between expression of the gene in the ovary of female and levels of mRNA transcripts in unfertilized egg for each treatment and mRNA abundance from unfertilized egg to embryos (shortly before hatch) related to hormonal treatment (CPE and SPE) and age of embryos (in hpf).

Gene	Relationship ovary – unfertilized eggs - CPE			Relationship ovary – unfertilized eggs - SPE			Model on unfertilized eggs and embryos			
	Equation	R^2	p-value	Equation	R^2	p-value	CPE (mean ± SE)	SPE (mean ± SE)	Hormonal treatment (p-value)	Age (p-value)
cdhr2	Y=0.01+0.67x	0.951	**<0.0001**	Y=-0.52+0.72x	0.954	**<0.0001**	9.31±0.89	6.18±0.88	**0.014**	0.208
cldng	Y=1.38-0.08x	0.003	0.847	Y=0.36+0.43x	0.086	0.290	0.54±0.04	0.41±0.08	**0.028**	**<0.0001**
dcbld1	Y=1.58-0.58x	0.195	0.076	Y=-5.03+0.58x	0.797	**<0.0001**	3.33±21.71	99.13±21.36	**0.0003**	0.958
dicer1	Y=1.25+0.32x	0.023	0.534	Y=1.03+0.25x	0.092	0.273	1.23±0.03	1.26±0.03	0.354	**<0.0001**
epcam	Y=1.26+0.17x	0.637	**0.0001**	Y=1.04+0.16x	0.906	**<0.0001**	5.88±0.70	8.85±1.63	**0.012**	**0.0003**
foxr1	Y=0.72+0.35x	0.084	0.243	Y=0.74+0.32x	0.318	**0.029**	0.67±0.05	0.48±0.11	**0.013**	**<0.0001**
cea	Y=0.30+0.64x	0.170	0.089	Y=0.11+0.83x	0.740	**<0.0001**	0.74±0.03	0.67±0.07	0.156	**<0.0001**
ccna1	Y=1.20+1.04x	0.239	**0.047**	Y=1.10+0.82x	0.692	**0.0001**	1.34±0.07	1.33±0.06	0.852	**<0.0001**
ccnb1	Y=3.37-1.76x	0.076	0.269	Y=0.55+0.90x	0.335	**0.030**	1.16±0.02	1.06±0.03	**0.013**	**<0.0001**
ccnb2	Y=2.67-1.35x	0.065	0.306	Y=1.02+0.16x	0.012	0.702	0.96±0.05	1.04±0.12	0.366	**<0.0001**
npm2	Y=2.97-0.45x	0.057	0.342	Y=0.88+0.15x	0.116	0.215	1.04±0.08	1.05±0.15	0.97	**<0.0001**
phb2	Y=1.43-0.02x	0.007	0.743	Y=0.93+0.05x	0.032	0.525	5.31±0.40	5.90±0.86	0.393	**<0.0001**
oct4	Y=-0.17+1.55x	0.336	**0.009**	Y=0.29+0.90x	0.567	**0.001**	0.80±0.03	0.92±0.08	**0.048**	**<0.0001**
sox2	Y=0.86+0.15x	0.167	0.103	Y=-0.16+0.55x	0.996	**<0.0001**	1070.99±89.07	1137.89±112.47	0.708	**<0.0001**
zar1	Y=3.60-0.11x	0.035	0.460	Y=1.12+0.009x	0.016	0.649	0.56±0.06	0.47±0.13	0.364	**<0.0001**
neurod4	Y=1.82-0.47x	0.079	0.242	Y=0.27+0.21x	0.878	**<0.0001**	1299.55±115.67	1279.14±217.08	0.378	**<0.0001**
neurog1	Y=1.37+0.63x	0.376	**0.007**	Y=1.31+0.14x	0.244	0.061	238.84±12.57	247.10±13.76	0.245	**<0.0001**
c3	Y=0.57+0.21x	0.231	**0.037**	Y=0.09+0.40x	0.646	**0.0003**	1150.66±125.19	829.36±285.06	0.314	**<0.0001**
igm	Y=0.32+0.15x	0.271	**0.022**	Y=0.03+0.40x	0.699	**0.0001**	0.53±0.06	0.85±0.14	**0.002**	**<0.0001**
il1β	Y=2.03+0.59x	0.012	0.651	Y=0.26+0.28x	0.282	**0.041**	0.75±0.06	0.78±0.06	0.768	**<0.0001**

Table 4. Relationship between mRNA abundance of specific genes in eggs and embryos at selected sampling points and offspring quality parameters for the two treatments (CPE and SPE) in European eel, *Anguilla anguilla*, including best fitting equation and significance levels for genes, where a significant effect was found for at least one of the treatments.

				CPE			SPE		
Gene	**Function**	**Relative abundance**	**Quality parameter**	**Equation**	**R^2**	**p-value**	**Equation**	**R^2**	**p-value**
dicer1	cell adhesion	2 hpf	Cleavage abnormalities	Y=52.67-5.57x	0.002	0.876	Y=94.24-60.74x	0.338	**0.023**
epcam	cell adhesion	2 hpf	Cleavage abnormalities	Y=67.28-9.22x	0.357	**0.024**	Y=24.95-0.30x	0.004	0.831
zar1	MZT	2 hpf	Cleavage abnormalities	Y=76.42-31.82x	0.149	0.155	Y=62.07-35.30x	0.324	**0.034**
zar1	MZT	8 hpf	Survival (48 hpf)	Y=-7.26+44.41x	0.326	**0.042**	Y=-15.3+47.07x	0.350	**0.020**
sox2	MZT	8 hpf	Survival (48 hpf)	Y=15.83+3.61x	0.729	**0.0002**	Y=7.89+17.17x	0.697	**0.0004**
foxr1	cell division	8 hpf	Survival (48 hpf)	Y=-23.32+45.92x	0.265	0.072	Y=-56.24+75.42x	0.495	**0.003**
cldn g	cell adhesion	8 hpf	Survival (48 hpf)	Y=0.75+41.59x	0.104	0.282	Y=-68.68+134.63x	0.364	**0.017**
phb2	normal mitochondrial function	8 hpf	Survival (48 hpf)	Y=-24.82+44.64x	0.204	0.122	Y=-32.78+49.99x	0.269	**0.047**
neurod4	Neurogenesis	8 hpf	Survival (48 hpf)	Y=20.60+0.96x	0.419	**0.017**	$Y=12.89+3.21x-0.04x^2$	0.516	**0.013**
neurog1	Neurogenesis	8 hpf	Survival (48 hpf)	Y=11.43+1.56x	0.672	**0.0006**	$Y=13.35+4.16x-0.05x^2$	0.509	**0.014**
Ccna1	cell cycle control	32 hpf	Hatch success	$Y=5.70+379.13x-478.20x^2$	0.524	**0.024**	Y=21.41+88.96x	0.408	**0.025**
npm2	nuclear organization	32 hpf	Hatch success	Y=32.02-19.88x	0.040	0.509	Y=11.19+151.72x	0.473	**0.013**
oct4	MZT	32 hpf	Hatch success	$Y=-5.12+545.65x-513.65x^2$	0.498	**0.032**	Y=21.11+115.98x	0.357	**0.040**
neurod4	Neurogenesis	32 hpf	Hatch success	Y=69.93-0.009x	0.048	0.473	$Y=82.06-0.03x+0.01x^2$	0.605	**0.015**
neurog1	Neurogenesis	32 hpf	Hatch success	Y=25.03+0.004x	0.00	0.931	Y=93.25-0.09x	0.601	**0.003**

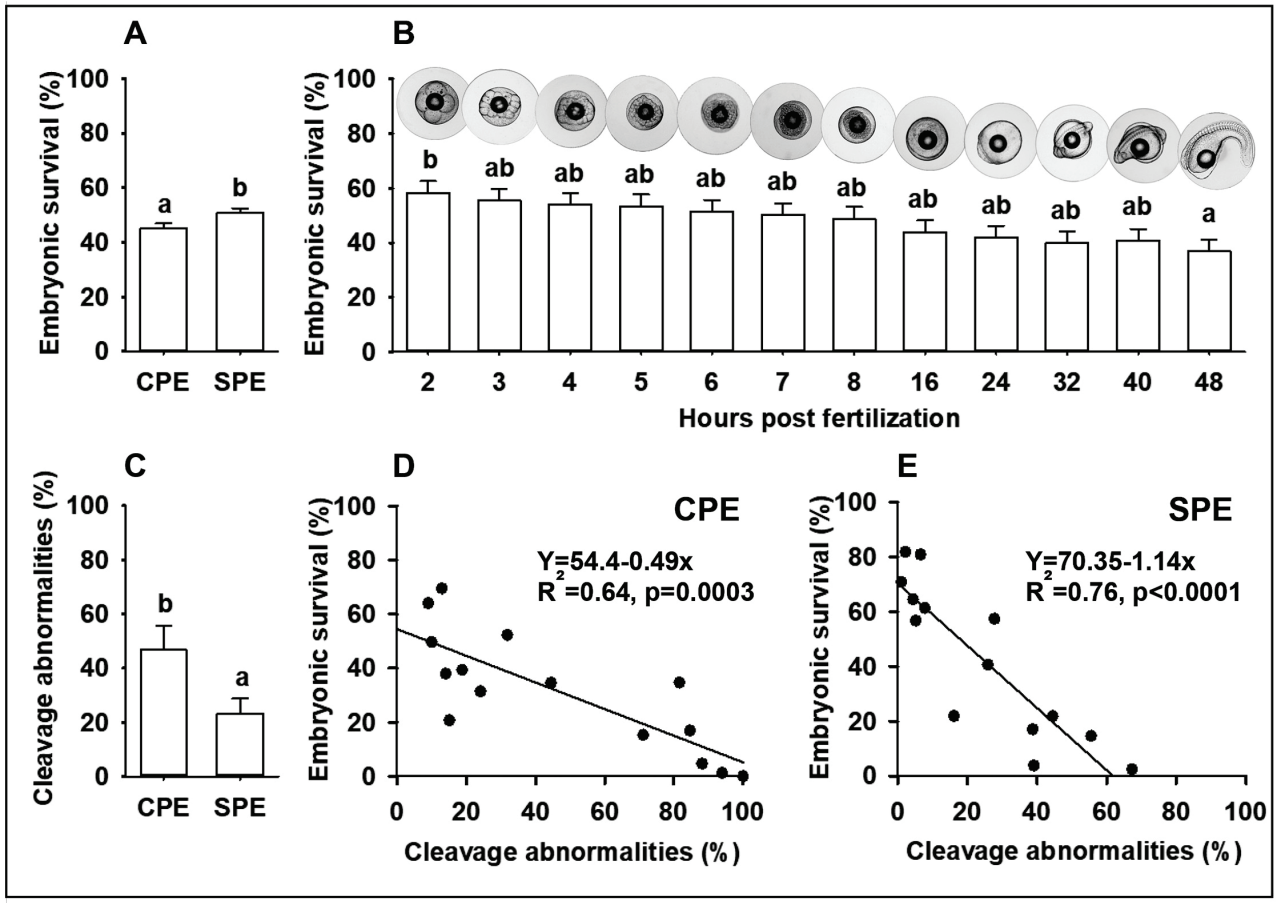

Fig 1. Embryonic survival and cleavage abnormalities in European eel, *Anguilla anguilla*. Embryonic survival in relation to (A) hormonal treatment and (B) age in hours post fertilization, (C) difference in proportions of cleavage abnormalities at 4 hpf among hormonal treatments and relationships between cleavage abnormalities at 4 hpf and embryonic survival at 48 hpf for (D) CPE and (E) SPE treatment. Values for bar plots represent means (± SEM) among embryos at each age and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

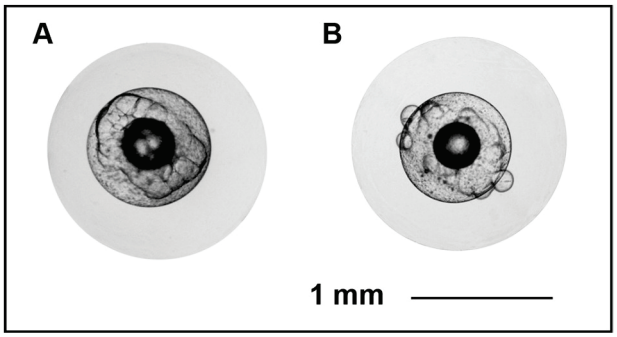

Fig 2. Example for cleavage abnormalities in European eel, *Anguilla anguilla*. Egg with (A) normal development and (B) occurrence of cleavage abnormalities at 4 hours post fertilization. Scale bar represents 1 mm.

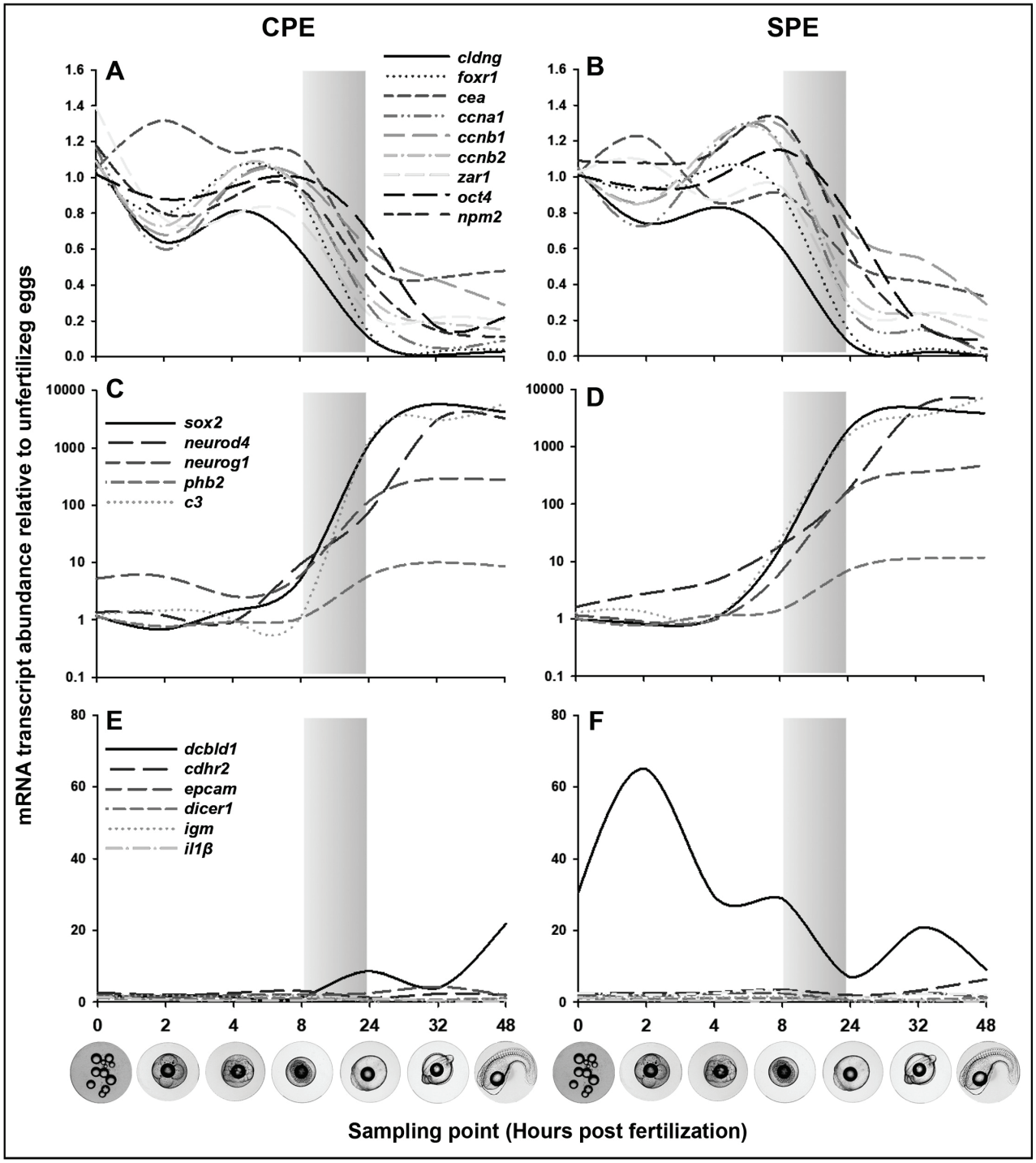

Fig 3. mRNA transcript abundance from unfertilized egg throughout embryonic development in European eel, *Anguilla anguilla*. Conceptual overview – Expression ($2^{-\Delta\Delta Ct}$) was calculated in relation to the average abundance in the unfertilized eggs of each gene. Relative abundance of *cldn g*, *foxr1*, *cea*, *ccna1*, *ccnb1*, *ccnb2*, *zar1*, *oct4*, *npm2* for (A) CPE and (B) SPE treatment. Relative abundance of *sox2*, *neurod4*, *neurog1*, *phb2*, *c3* for (C) CPE and (D) SPE treatment. Relative abundance for *cdhr2*, *dcbld1*, *epcam*, and *dicer1*, *igm*, *il1β* for (E) CPE and (F) SPE treatment. Bars represent timeframe of maternal-to-zygotic transition (MZT).

Paper III: Sex steroid dynamics and mRNA transcript profiles of growth and development related genes during embryogenesis following induced follicular maturation in European eel

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Manuscript

Sex steroid dynamics and mRNA transcript profiles of growth and development related genes during embryogenesis following induced follicular maturation in European eel

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Abstract

Maternally derived hormones and mRNA transcripts control early embryonic development in teleost fish. The former includes steroids, such as estradiol-17β (E2), testosterone (T), 11-ketotestosterone (11-kt), 17α,20ß-dihydroxy-4-pregnen-3-one (DHP), and cortisol, which also play an important role in fish reproduction. In European eel, *Anguilla anguilla*, vitellogenesis in female broodstock is commonly induced by exogenous gonadotropins in form of salmon (SPE) or carp pituitary extract (CPE), while follicular maturation is initiated by a priming dose of PE followed by administration of DHP as a maturation inducing hormone. In this regard, the main purpose of the present study was to evaluate effects of induced follicular maturation on reproductive success in European eel, focusing on maternal transfer and dynamics of sex steroids and mRNA transcripts of growth and development related genes throughout embryogenesis. Detected concentrations of E2, T and DHP in maternal blood plasma explained the observed variation in the unfertilized eggs, while concentrations of E2 and DHP in eggs and embryos were negatively related to quality parameters measured as fertilization success, cleavage abnormalities, embryonic survival, and hatch success. Concomitant, mRNA transcript abundance analysis included genes involved in stress response (*hsp70, hsp90*), somatotropic axis (*gh*, *igf1*, *igf2a*, *igf2b*), as well as lipid (*cpt1a*, *cpt1b*, *pigf5*) and thyroid metabolism (*dio1*, *dio2*, *dio3*, *thrαb*, *thrβa*, *thrβb*). These mRNA transcripts were present in the unfertilized eggs at a variable level. For the majority of genes, an increase in mRNA abundance happened after the activation of the embryos own genome transcription during the maternal-to-zygotic transition. Moreover, mRNA abundance of *dio1*, *cpt1a* and *cpt1b* throughout embryogenesis was related to their developmental competence. Finally, the mRNA abundance of *dio3* was associated to E2 concentrations and the mRNA abundance of *thrαb* was associated to T concentrations in the unfertilized eggs indicating a possible interplay between the thyroid and steroid hormone systems. Taken together, maternal plasma concentrations of E2 and DHP were

reflected in the eggs with high concentrations of these steroids in the eggs impacting embryonic developmental competence. These results indicate that induction of follicular maturation influences concentrations of E2 and DHP in egg quality and embryogenesis. In contrast, mRNA transcripts of only two genes were directly associated with embryonic quality parameters, while expression patterns for genes involved in growth, development and metabolism underlined their role during later embryogenesis.

Keywords

Assisted reproduction, radioimmunoassay, gene expression, qPCR, hormone receptor

1. Introduction

In teleosts, maternally derived constituents play an important role during early embryonic development (Brooks et al., 1997; Lubzens et al., 2010). This includes components such as proteins, lipids, maternal mRNA, but also lipophilic hormones that are incorporated into the egg yolk during vitellogenesis and transferred to the offspring influencing their developmental competence (Brooks et al., 1997; Lubzens et al., 2010; Tokarz et al., 2015). The dynamics of sex hormones in fish are regulated by the hypothalamus–pituitary–gonadal axis (HPG), where the gonadotropin-releasing hormone (GnRH) regulates the synthesis and release from the pituitaries of the two gonadotropin hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Levavi-Sivan et al., 2010; Nagahama and Yamashita, 2008). These hormones act on the gonads to control gamete development and the production of sex steroids.

Overall, steroid hormones are involved in numerous physiological processes, such as metabolism, immune response, reproduction, embryonic development, and sex determination and differentiation (Tokarz et al., 2015). Until now, very little knowledge exists regarding the impact of

steroids on embryogenesis of teleost species (Tokarz et al., 2013). Nonetheless, the activation of the hypothalamus–pituitary–interrenal (HPI) axis and *de novo* steroid synthesis in teleosts is likely not initiated until after hatch, thus maternally derived steroids are expected to guide embryonic development (Nesan and Vijayan, 2013). In fact, the presence of different types of steroids in unfertilized eggs and throughout embryogenesis has been evidenced in several fish species, e.g. zebrafish (Alsop and Vijayan, 2008; Busby et al., 2010; Nesan and Vijayan, 2012; Pikulkaew et al., 2010), Coho Salmon, *Oncorhynchus kisutch* (Feist et al., 1990), Arctic charr, *Salvelinus alpinus* (Khan et al., 1997), three-spined stickleback, *Gasterosteus aculeatus* (Paitz et al., 2015), medaka, *Oryzias latipes* (Iwamatsu et al., 2006), tilapia (Rothbard et al., 1987), Eurasian perch, *Perca fluviatilis* (Rougeot et al., 2007), white sturgeon, *Acipenser transmontanus* (Simontacchi et al., 2009), and Japanese flounder, *Paralichthys olivaceus* (de Jesus et al., 1991). Overall, steroid concentrations are present in the unfertilized eggs and show a steady decline after fertilization and towards hatch. However, maternal exposure to cortisol in experiments on Atlantic salmon, *Salmo salar* led to detrimental effects on the offspring, such as increased mortality and malformations and decelerated yolk sac utilization (Eriksen et al., 2006; 2007).

The importance of steroids during different phases of the oogenesis has also been shown for anguillid species (Burgerhout et al., 2016; da Silva et al., 2016; Kazeto et al., 2011). The European eel, *Anguilla anguilla*, shows a silver prepubertal stage in which a strong dopaminergic inhibition prevents sexual maturation in their continental habitats (Vidal et al., 2004). During the long migration to their oceanic spawning grounds in the Sargasso Sea, this inhibition must be released, however, the exact mechanisms are unknown, as no maturing or spawning female has ever been found in the wild. In captivity, sexual maturation does not occur unless hormonal treatments are applied, which includes the administration of exogenous gonadotropins (Dufour et al., 1983). Pressured by a strong decline of natural stocks, efforts to establish a sustainable aquaculture have

increased, leading to a stable production of viable European eel eggs and first-feeding larvae (Butts et al., 2016; Politis et al., 2018c; Tomkiewicz et al., 2019). Nonetheless, high variability in egg quality and offspring performance call for further research to enhance egg and larval quality aiming for a closed-cycle production. During vitellogenesis, the importance of estradiol-17β (E2), 11-ketotestosterone (11-kt), and 17α,20ß-dihydroxy-4-pregnen-3-one (DHP) was shown in Japanese eel, *A. japonica* (Ijiri et al., 1995; Kazeto et al., 2011; Matsubara et al., 2005). While E2 and 11-kt play a primary role during previtellogenic and vitellogenic growth of oocytes, DHP is important for the induction of follicular maturation. Also in European eel, the steroid profile during different phases of the sexual maturation and their importance for successful final sexual maturation has been described (Burgerhout et al., 2016; da Silva et al., 2016). Here, levels of E2, testosterone (T) and 11-kt increase throughout maturation. While E2 shows a constant increase from pre-vitellogenic stage until germinal vesicle breakdown, levels of T and 11-kt increase throughout vitellogenesis but show a decrease towards final maturation (da Silva et al., 2016).

Likewise, maternally derived mRNA is deposited into the egg during oocyte development affecting early stages of embryonic development (Lubzens et al., 2017). Here, specific mRNA transcripts may have profound impacts on egg quality and early developmental competence (Aegerter et al., 2004; Lanes et al., 2013; Mommens et al., 2010; Rozenfeld et al., 2016). The embryo takes over control by its own genome activation during the maternal-to-zygotic transition (MZT) (Newport and Kirschner, 1982). This takes place during the mid-blastula transition, which in European eel occurs ~ 10 hours post fertilization (hpf) at 18°C (Sørensen et al., 2016). The expression profiles of growth and development related genes involved in stress/repair, somatotropic axis, as well as thyroid and lipid metabolism have been shown for European eel larvae (Politis et al., 2017; 2018a; 2018c). In addition, there are first indications that maternal mRNA also affects embryogenesis in *A. anguilla* (Rozenfeld et al., 2016).

To that end, the current study examined the possible maternal transfer of sex steroids as well as the temporal changes during embryogenesis in European eel. Moreover, the effect of steroid levels on egg quality and embryonic development was explored by categorizing offspring into high, medium, and low quality groups. Furthermore, a transcriptional expression ontogeny during embryonic development was conducted for genes involved in stress response (*hsp70, hsp90*), somatotropic axis (*gh*, *igf1*, *igf2a*, *igf2b*), as well as lipid (*cpt1a*, *cpt1b*, *pigf5*) and thyroid metabolism (*dio1*, *dio2*, *dio3*, *thrαb*, *thrβa*, *thrβb*).

2. Materials and Methods

2.1. Ethics statement

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 approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696). Individual fish were anesthetized before tagging, biopsy, and stripping of gametes, and euthanized after stripping (females) or at the end of the experiment (males) using an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg L^{-1}, Sigma Aldrich, Germany). Larvae were anesthetized and euthanized using tricaine methanesulfonate (MS-222, 25 mg L^{-1}, Sigma Aldrich, Germany).

2.2. Broodstock management and gamete production

Data were collected throughout two consecutive trials in 2016 and 2017 with female silver eels caught during down-stream migration. In 2016, 13 female silver eels (length = 76 ± 5.97 cm; weight = 897 ± 223.38 g) were caught at Lower Bann, Toomebridge, Northern Ireland, while in 2017, 11 female eels (length = 63.55 ± 6.77 cm; weight = 518.36 ± 165.18 g) were caught at a

freshwater Klitmøller Å, Lake Vandet, Denmark. The female eels were transported to the EEL-HATCH experimental facility at DTU in Hirtshals, Denmark, using an aerated freshwater tank. Here, fish were randomly distributed into replicated 1150 L tanks, connected to two Recirculating Aquaculture Systems (RAS), at a density of 10-15 females per tank.

In both trials, male eels originated from Stensgård Eel Farm, where they were raised from glass eels on a formulated diet (DAN-EX 2848, BioMar A/S, Denmark) at a temperature of ~23 °C. In 2016, experiments comprised 60 male eels (length = 38.2 ± 2.1 cm, weight = 105.5 ± 15.3 g), and in 2017, 88 males (length = 38.5 ± 2.1 cm, weight = 114.7 ± 15.8 g). After transport to the facility, males were randomly distributed in four tanks (485 L) connected to a RAS unit at a density of ~15-20 eels per tank.

For acclimatization, salinity was gradually increased from 10 to 36 PSU over 14 days using Blue Treasure Aquaculture Salt (Qingdao Sea-Salt Aquarium Technology Co. Ltd. Qingdao, China). Subsequently, each individual was tagged with a passive integrated transponder (PIT tag) in the dorsal muscle, while initial length and weight were recorded. At the facility, male and female broodstock were reared at ~20 °C and ~36 PSU under 12 h - 12 h light regime, with a 30 min twilight in the morning and evening to resemble the Sargasso Sea photoperiod.

Vitellogenesis in the female broodstock was induced by weekly intramuscular injections of salmon pituitary extract (SPE) at 18.75 mg kg^{-1} initial body weight (BW) for 11-21 weeks (Tomkiewicz, 2012). According to body-weight increase and oocyte developmental stage, monitored by biopsies, an additional injection of the respective hormone was given to each female as a primer. After 12-24 hours, the female received an injection of DHP (Sigma-Aldrich, St. Louis, MO, USA) at 2 mg kg^{-1} current BW to stimulate follicular maturation and ovulation (Ohta et al., 1996). Male eels received weekly injections of human chorionic gonadotropin (Sigma-Aldrich,

Missouri, USA) at 150 IU/fish (Tomkiewicz, 2012). Prior to spawning, milt from 3-5 males was collected, sperm concentration standardized (Sørensen et al., 2013), and the dilution kept in an immobilizing medium (Peñaranda et al., 2010a). Eggs were strip-spawned and fertilized using a standardized sperm to egg ratio (Butts et al., 2014; Sørensen et al., 2015). After five min, eggs were transferred to 20 L buckets filled with ~15 L reverse osmosis water salted to ~36 PSU with Blue Treasure (Qingdao Sea-Salt Aquarium Technology Co., Ltd., Qingdao, China) 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. Eggs/embryos were taken from the floating layer of the separation bucket and subsequently incubated in 10 1 L glass beakers (~5000 eggs/embryos per L), each 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). Subsequent rearing occurred in a temperature incubator at 18°C (Politis et al., 2018a) and 36 PSU. Additionally, 6 × 200 mL sterile tissue culture flasks filled with FUV seawater and supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) were stocked with eggs/embryos and incubated as above. Here, 3 flasks stocked with ~2500 eggs/embryos were used to follow embryonic development and 3 flasks stocked with ~600 eggs/embryos were used to analyze hatch success.

2.3. Data collection

Immediately after spawning, blood plasma was taken from the caudal vessel of the anaesthetized female and centrifuged (10 minutes, 4360 RPM, 4 RCF). Plasma was distributed into Eppendorf vials and stored at -20°C. The female was then euthanized using an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg L^{-1}, Sigma Aldrich, Germany) and dissected. Ovarian tissue was sampled in Eppendorf vials filled with RNAlater (Sigma Aldrich, Germany). The samples were kept in the fridge at 4°C for 24 hours and subsequently stored at -20°C. Moreover, unfertilized eggs were sampled for steroid analyses (4 × 0.25 ml, frozen at -20°C) and gene

expression (2 × 0.25 ml, kept in RNAlater in the fridge at 4°C for 24 hours and subsequently stored at -20°C). Embryos were sampled at 2, 4, 6, 8, 24, 32, 48 hours post fertilization (hpf). Here, samples for steroid analyses (4 × 0.25 ml) were kept frozen at -20°C, while samples for gene expression stored as described above. For quantification of fertilization success, occurrence at cleavage abnormalities [4 hours post fertilization (hpf)] and embryonic survival (48 hpf), digital images were taken using a Nikon Eclipse 55i microscope equipped with a Nikon digital sight DS-Fi1 Camera. 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 floating eggs. Cleavage abnormalities at 4 hpf were determined by counting the number of eggs with regular and irregular cell cleavages obtained from floating eggs. Embryonic survival at 48 hpf was measured by counting the number of dead and alive eggs and expressed as a percentage (obtained from floating eggs). Hatch success was expressed as the number of hatched larvae divided by the total number of fertilized eggs.

2.4. Steroid analyses

Concentrations of DHP, E2, T, 11-kt and cortisol were measured in female blood plasma, unfertilized eggs, and embryos by means of radioimmunoassay (RIA), as described by Schulz (1985). Assay characteristics and cross-reactivities of E2 and T antisera have previously been examined by Frantzen et al. (2004) and validated for eel plasma by Mazzeo et al. (2014). For DHP, it has been described by Tveiten et al. (2010b) validated for eel plasma by Peñaranda et al. (2010b). The cross-reactivities of a new 11-kt antiserum have been described by Johnsen et al. (2013) and validated for eel plasma by Baeza et al. (2015), while for cortisol previous descriptions can be found in Tveiten et al. (2010a). In short, free (i.e. non conjugated) steroids were extracted from the sample (200 µL blood plasma or 360 mg eggs and embryos) with 4 mL diethylether under vigorous shaking for four minutes. Subsequently, the aqueous phase was frozen in liquid nitrogen and

separated from the organic phase, which was then transferred to a new glass tube kept in a water batch at 45°C until all ether was evaporated. The steroids were reconstituted by adding 900 µL of RIA-buffer and then assayed for each sex steroid.

To validate the recovery of each steroid, triplicates of embryo samples were spiked with a known amount of radiolabeled steroids (20-30 000 cpm (counts per minute)) of each steroid and subsequently underwent the extraction procedure, as described above and assayed for the respective steroid. The extraction efficiencies were 87.18±1.67 % for DHP, 77.91±1.31 % for T, 71.15±1.09 % for E2, 68.70±2.15 % for 11-kt, and 75.48±2.04 % for cortisol. The extraction efficiency factor was included in the scaling factor, when calculating concentrations of the different steroids. Moreover, a dilution curve at 9 different dilutions was made and found to be parallel to the standard assay curve.

2.5. Gene expression

RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Germany) according to manufacturer's instructions. RNA concentration and purity was analyzed by spectrophotometry using Nanodrop One (Thermo Fisher Scientific, USA). 1 µg RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quantabio, Germany), following the manufacturer's instructions including a DNase step using PerfeCta DNase I (Quantabio, Germany). Primers of 19 genes were retrieved from previous studies (Table1) or designed using primer 3 software v 0.4.01 based on the coding sequence (cds) based on the predicted annotation of the European eel reference genome (Henkel et al., 2012) or with the available cds of *Anguilla japonica* when the annotation was not available. All primers were tested in silico for specificity using blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, from all samples the expression of 19 genes (Table1) was analyzed with two technical replicates using the qPCR BiomarkTM HD system (Fluidigm) based on 96.96 dynamic arrays (GE plates), as previously described (Miest et al., 2016).

In brief, a pre-amplification step was conducted with a 500 nM pool of all primers in PreAmp Master Mix (Fluidigm) and 1.25 µL cDNA per sample run in a thermocycler for 2 min at 95 °C; 10 cycles: 15 s each at 95 °C and 4 min at 60 °C. Obtained PCR products were diluted 1:5 with low EDTA-TE buffer. The preamplified product was loaded onto the chip with SsoFast-EvaGreen Supermix Low Rox (Bio-Rad) and DNA-Binding Dye Sample Loading Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 µM in Assay Loading Reagent (Fluidigm) and low EDTA-TE Buffer. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60 °C. qBase + software verified stability of housekeeping gene expression throughout analyzed samples (M < 0.4; according to Hellemans et al. (2008)). Gene expression was normalized (ΔCt) to the geometric mean of the four most stable housekeeping genes (*ccna2*, *cei*, *thαa*, *igfr-1b*). Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method, in relation to a random unfertilized egg sample, according to (Livak and Schmittgen, 2001).

2.6. Statistical Analyses

Data were analyzed 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 $\log_{10}$ transformed. Alpha was set at 0.05. Tukey's analysis was used to compare least-squares means between groups. 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).

For female and quality parameters, ANOVA models were run testing the effect of broodstock series (2016, 2017), quality groups and their interaction. Here, no significant interactions were found and therefore removed. While initial length ($p = 0.001$) and weight ($p = 0.002$) differed between female broodstock from the two locations, quality parameters were similar

among female broodstock, including fertilization success ($p = 0.320$), cleavage abnormalities ($p = 0.560$), embryonic survival at 48 hpf ($p = 0.095$), and hatch success ($p = 0.280$). Therefore, data from the two broodstock series were pooled. The division into three quality groups (high, medium, low) was based on fertilization success, the occurrence of cleavage abnormalities, survival at 48 hpf, and hatch success.

Comparison of plasma steroid concentrations in females of the different quality groups was analyzed using a series of one-way ANOVA models. DHP and E2 concentrations in the unfertilized eggs and embryos were analyzed using a repeated measures two-way ANOVA testing the main effects of quality group (high, medium, low) and age (unfertilized egg, 2, 4, 6, 8, 24, 48 hpf) as well as their interaction. 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). Comparison of T, 11-kt and cortisol concentrations of the different quality groups in the unfertilized eggs and at 2 hpf were analyzed separately using a series of one-way ANOVA models. mRNA abundance data were analyzed using a repeated measures two-way ANOVA testing the effect of quality group (high, medium, low) and age (unfertilized egg, 2, 4, 6, 8, 24, 32, 48 hpf) as well as their interaction. A significant interaction was only found for *cpt1a*, where the model was decomposed into a series of one-way ANOVA models testing the effect of quality at each age. Moreover, relationship between steroid concentrations in the female blood plasma and unfertilized eggs, between mRNA abundance in the female ovarian tissue and the unfertilized eggs as well as between steroid concentrations and mRNA abundance levels were tested for linear, quadratic and cubic relationship. In case more than one regression function was significant, F-statistics were used to evaluate best fit.

3. Results

Overall, 16 out of 24 females successfully produced eggs and were included in the analysis. An overview over all females and their success is summarized in Table 2. Females were categorized into high, medium and low quality groups depending on developmental competence of their eggs.

3.1. Steroid concentrations

DHP concentrations in female plasma after stripping did not differ significantly between quality groups, although a trend towards higher concentrations in the low category was observed ($p = 0.057$; Fig. 1A). In unfertilized eggs and embryos, DHP concentrations decreased during embryonic development with lowest concentrations close to hatch ($p < 0.0001$; Fig. 1D). Here, concentrations of DHP were higher in the medium and low quality groups ($p < 0.0001$; Fig. 1B, C).

Female post-stripping E2 plasma concentrations did not differ between quality groups. On the other hand, E2 concentrations were found to be higher in the eggs and embryos from the low quality group ($p = 0.0003$), while the high and medium group did not differ (Fig 1G). Again, concentrations decreased over time with highest levels in the unfertilized egg and decreasing towards hatch ($p < 0.0001$; Fig. 1F, H).

Concentrations of T, 11-kt, and cortisol are summarized in Table 3. Here, female post-stripping T plasma concentrations did not differ between quality groups ($p = 0.381$), although a trend for higher concentrations in the low category was observed. In the unfertilized eggs, lowest concentrations were found in the high quality group compared to the medium and low quality group, however, these differences were non-significant ($p = 0.196$). However, T concentrations in embryos at 2 hpf differed ($p = 0.027$) with concentrations in the low quality group being significantly higher than in the medium quality group, while the concentrations in the high quality

group did not differ. Overall, concentrations at 2 hpf were close to the assay detection limits and no further measurements throughout embryonic development were made.

Female post-stripping 11-kt plasma concentrations also did not differ between quality groups ($p = 0.594$). Similarly, concentrations in the unfertilized eggs were similar among quality groups and overall quite low ($p = 0.668$). Again, concentrations at 2 hpf did not differ between quality groups ($p = 0.893$). Here, E2 concentrations were close to assay detection levels and no further analyses were made.

Female post-stripping cortisol plasma concentrations did not differ between quality groups ($p = 0.597$). Moreover, concentrations in the unfertilized eggs were overall low and also did not differ among groups ($p = 0.733$). At 2 hpf, cortisol was below the assay detection levels in most of the embryos, therefore no statistical analyses and no further analyses were performed.

Female post-stripping plasma steroid concentrations were associated to concentrations in the unfertilized egg and best described by a positive linear regression for DHP (Fig 2A), E2 (Fig 2B), and T (Fig 2C). No significant association was found for 11-kt (Fig 2D) and cortisol (Fig 2E).

3.2. mRNA transcript abundance and gene expression patterns

Overall, different mRNA abundance patterns over time were observed for gene groups, with most of the genes showing increasing mRNA abundance after the MZT. Genes involved in stress/repair mechanisms showed relatively low mRNA abundance during early development and peaked towards hatch, i.e. 32 hpf for *hsp90* and 48 hpf for *hsp70* (Fig. 3A). Similar patterns were observed for genes of the somatotropic axis (*gh*, *igf1*, *igf2a*, *igf2b*), which displayed low mRNA abundance during the first eight hours and rapid increases after the MZT at 24 hpf (Fig. 3B). Genes involved in lipid metabolism had low mRNA abundance during early development (Fig. 3C). Here, *cpt1a* increased rapidly at 48 hpf, while *cpt1b* already increased at 24 and 32hpf. Also, *pigf5*

increased towards hatch, however to a lower extent. Generally, genes involved in thyroid metabolism (*dio1*, *dio2*, *dio3*, *thrαb*, *thrβa*, *thrβb*) also showed relatively low mRNA abundance during early development, with the exception of *dio1*. The most rapid increases were found for *dio2* and *dio3* at 32 and 48 hpf. mRNA abundance of *thrαb*, *thrβa*, *thrβb* only showed slight increases towards hatch (Fig. 3D).

The mRNA level in the female ovary was associated with the mRNA abundance in the unfertilized eggs for eight genes (*hsp70*, *hsp90*, *cpt1a*, *cpt1b*, *pigf5, dio1*, *thrαb*, *thrβa*). Here, associations were best described by linear regressions for *hsp70* ($Y = 0.34 + 0.65x$; $R^2 = 0.979$; $p < 0.0001$), *hsp90* ($Y = 0.09 + 0.42x$; $R^2 = 0.345$; $p = 0.021$), *dio1* ($Y = 70.76 + 0.26x$; Fig 5C), *thrαb* ($Y = 0.82 + 0.19x$; $R^2 = 0.277$; $p = 0.044$), *thrβa* ($Y = 0.16 + 0.91x$; $R^2 = 0.617$; $p = 0.0005$), *cpt1a* ($Y = 0.01 + 1.0x$; Fig 4H), *cpt1b* ($Y = 0.23 + 0.15x$; Fig. 4F), and *pigf5* ($Y = 0.33 + 0.43x$; $R^2 = 0.577$; $p = 0.001$). The mRNA abundance of three genes differed between quality groups (Fig. 4). Here, *dio1* mRNA levels were relatively stable throughout embryonic development but were significantly lower in the low quality group compared to the medium and high quality group (Fig. 4A,B). The mRNA abundance of *dio1* in the ovary was associated to the abundance in the unfertilized egg (Fig. 4C). The mRNA levels of *cpt1b* were highest in the high and medium quality group being significantly lower in the low quality group (Fig. 4D). mRNA abundance of this gene was relatively low in the unfertilized egg and stayed stable until 8hpf. Subsequently, a rapid increase was observed at 24 hpf increasing further towards hatch. The mRNA abundance in the ovary was associated to the abundance in the unfertilized egg. The mRNA abundance of *cpt1a* showed a significant interaction between quality groups and hpf ($p = 0.001$) and was therefore analyzed separately at each sampling point. Here, mRNA abundance until 24 hpf remained relatively stable and did not differ between quality groups (Fig 4G). Hereafter, levels increased at 32 and 48 hpf. Moreover, at 32 hpf higher levels in the medium quality group were found compared

to the high quality group, while the low quality group was intermediate. Again, mRNA abundance of *cpt1a* in the female ovary was positively associated to levels in the unfertilized eggs (Fig. 4H).

3.3. Steroid concentrations – mRNA transcript abundance

The mRNA abundance of each analyzed gene was tested for association with each steroid concentration in the unfertilized eggs. Here, an association between thyroid hormone receptors and steroid levels in the unfertilized eggs was found. E2 concentrations were positively associated with the relative mRNA abundance of *dio3* (Fig. 5A). Moreover, the T concentrations were negatively associated with the relative mRNA abundance of *thrαb* (Fig. 5 B, C).

4. Discussion

4.1. Steroids

DHP is known to be the most effective maturation-inducing hormone (MIS) in many teleost species (Nagahama, 1983; Nagahama and Yamashita, 2008). In anguillid species, the injection of DHP is used to induce final maturation once oocytes reach the migratory nucleus stage (Ohta et al., 1997; Tomkiewicz, 2012). The present study showed maternal transfer of DHP to the unfertilized egg with the steroid content being reflected in the maternal post-stripping plasma. Egg DHP concentrations decreased during embryonic development indicating steroid metabolization by the embryo. Furthermore, high concentrations of DHP appeared to influence embryonic development negatively. The presence of maternally derived DHP has been shown in eggs of Coho salmon (Feist et al., 1990), Arctic charr (Khan et al., 1997), and three-spined stickleback (Paitz et al., 2015). In agreement with our results, a strong decline following fertilization was found for all three species indicating metabolization and elimination of this steroid during early embryonic stages. In Arctic charr, it was suggested that at least two enzyme systems were present, cytochrome P450 C_{21} side

chain cleavage converting progesterone (P_4) to 17-hydroxyprogesterone (17OHP) and further to androstenedione (A_4) and secondly, 11βhydroxylase that convert A_4 to 11-oxyandrogens (Khan et al., 1997). This may be of advantage for the embryos, as these products have a potentially lower biological activity than steroids such as E2 and T (Khan et al., 1997). This is further supported by results from a study on Coho salmon, where DHP concentrations were higher in non-viable eggs compared to viable eggs (Feist et al., 1990). Another possible explanation for the association between high egg DHP concentrations and poor egg quality might be that related to premature recruitment into follicular maturation. Unpublished in vitro studies show that European eel ovarian follicles are able to metabolize DHP into inactive DHP-sulphate (H. Tveiten, pers. comm.) which may be a mechanism to protect the oocyte from DHP overexposure and a premature entry into follicular maturation. This metabolization, or inactivation mechanism, of MIS during follicular maturation is also found in other marine teleosts (Scott et al., 1997; Scott and Sorensen, 1994; Tveiten et al., 2000, 2010b). The high (150-200 ng/ml) DHP plasma concentrations associated with artificial DHP induced follicular maturation in eel, might supersaturate this inactivation system resulting in increased DHP accumulation in the oocyte/egg. Thus, it can be speculated that low quality eggs with high DHP concentrations and of low quality, may have been recruited into follicular maturation at a too early stage of development, negatively influencing their further development (i.e. fertilization success, occurrence of cleavage abnormalities, embryonic survival).

Concentrations of E2, T and 11-kt in eggs and embryos have been studied in several species. For instance, a maternal transfer with presence of E2, T and 11-kt in the unfertilized eggs has been suggested in Eurasian perch, *Perca fluviatilis* (Rougeot et al., 2007), Coho salmon (Feist et al., 1990), tilapia (T and E2) (Rothbard et al., 1987), white sturgeon, *Acipenser transmontanus* (T, E2 and cortisol) (Simontacchi et al., 2009), medaka, *Oryzias latipes* (T and E2) (Iwamatsu et al., 2006), and threespined stickleback (T and E2) (Paitz et al., 2015). In the present study, E2, T and 11-kt

were present in the unfertilized eggs of European eel. More so, the concentration in the unfertilized eggs of E2 and T were associated with the concentration in female post-stripping plasma, indicating a maternal impact on steroid levels in the eggs. Concentrations of T and 11-kt were almost non-detectable in fertilized eggs and there were no apparent relationships with egg quality, indicating fast metabolization. However, E2 was higher in low quality embryos suggesting the importance of metabolic degradation of E2 throughout embryogenesis to allow normal development in European eel. Moreover, elevated E2 might reflect that oocytes may still have been vitellogenic and support the above discussion about a too early entry into follicular maturation. This is also in accordance with findings in Coho salmon, where concentrations of E2 were higher in non-viable eggs compared to viable eggs (Feist et al., 1990). However, the exact role of E2 during these early stages of embryonic development remains to be investigated. Recently, studies on estrogen receptor expression have elucidated their important role during follicular maturation in European eel (da Silva et al., 2018). In this study, the nuclear receptors *esr1* and *esr2a* were expressed at the time of SPE priming and DHP injection but hardly in the ovulated eggs. While mRNA transcripts of the membrane receptor *gpera* were present in the unfertilized eggs, levels of *gperb* were below the detection threshold. However, at the time of DHP injection, a higher expression of *gperb* was found in females producing low quality eggs. This observation may support findings in the present study where high concentrations (signaling) of E2 appear to have negative effects on egg quality. Further investigations may reveal if there is a direct relationship between receptor abundance and E2 with egg quality. Androgen receptor (*ara*, *arb*) levels increase throughout ovarian development in both European eel (Peñaranda et al., 2014) and Japanese eel (Tosaka et al., 2010). However, in the latter, mRNA transcripts in the ovulated eggs were very low (*ara*) or undetectable (*arb*) indicating only limited maternal transfer of mRNA.

Maternal stress may lead to increased deposition of cortisol into the egg with possible implications on the embryonic developmental competence (Nesan and Vijayan, 2012). In this study, cortisol was found in the blood plasma of the female in the 15-25 ng/ml range but only low concentrations (and no association with plasma concentrations) were found in the unfertilized eggs. Cortisol concentrations reached detection levels already at 2 hpf and no relationship with egg quality was observed. In other fish species, protective measures to prevent excess cortisol entering the eggs may be related to upregulation of cortisol inactivating enzymes, such as 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) (Faught et al., 2016). Whether a similar mechanism may be present in European eel ovaries needs, however, further investigations.

4.2. mRNA transcript abundance

Overall, this study investigated the mRNA transcript profiles of genes assumed to be important for embryonic development in European eel. The genes selected are known to be involved in stress/repair responses, growth and development, as well as lipid and thyroid metabolism. In our study, most of these genes showed low mRNA abundance before MZT and an increase in expression upon activation of the embryos own genome.

4.2.1. Genes related to cellular stress

Heat shock proteins, such as *hsp70* and *hsp90* function as chaperones and are recognized to be upregulated in response to cellular stress (Roberts et al., 2010). In teleost embryos, it has been shown that *hsp* levels are both affected by developmental age (Blechinger et al., 2002; Lanes et al., 2012) and cellular stress (Hallare et al., 2005; Sales et al., 2019; Uchimura et al., 2019; Yeh and Hsu, 2002). In the present study, *hsp90* peaked at 32 hpf with a subsequent decline towards hatch indicating a role in embryonic development and possible stress response during organogenesis, while *hsp70* showed a slight increase towards hatch indicating a possible role for hatched larvae. In

European eel larvae, both genes are affected by environmental parameters, such as temperature and salinity (Politis et al., 2017; 2018a), which remains to be investigated for embryos. *Hsp70* is required to prevent stress-induced cell death (Mosser et al., 2000) and *hps90* is essential for cell viability and normal embryonic development in zebrafish embryos allowing intracellular signaling and proliferation and/or differentiation (Lele et al., 1999). However, in the current study, no difference in concentrations between quality groups was observed for embryos being reared under the same conditions.

4.2.2. Genes related to the somatotropic axis

Genes involved in the somatotropic axis take place in numerous processes including reproduction and growth during embryonic development (Reinecke et al., 2005; Reinecke and Collet, 1998). In the present study, all genes involved in growth and development showed a similar pattern with strong increases after the MZT indicating that the somatotropic axis is functional and may play a role already during embryonic development in European eel. Additionally, no differences between quality groups were observed, which is in agreement with a previous study on eel with no differences in the expression of *igf2a and igf2b* between high and low hatch groups (Rozenfeld et al., 2016). This, however, appears to be species specific, as *igf1*, *igf2*, and *igfr1b* have been positively associated with embryonic survival in rainbow trout (Aegerter et al., 2004; 2005). The expression patterns of *igf* and *gh* during embryogenesis have been shown for various species, such as zebrafish (Li et al., 2014; Zou et al., 2009), maraena whitefish, *Coregonus maraena* (Nipkow et al., 2018), gilthead seabream, *Sparus* aurata (Perrot et al., 1999), and seabass, *Dicentrarchus labrax* (Besseau et al., 2013). Similarly, these patterns appear to be species specific as well and for some species expression has only been observed after hatch, as in the closely related Japanese eel (Ozaki et al., 2006). Moreover, *gh* and *igf* may show differential expression patterns indicating that they *igf* expression is not *gh*-dependent, yet, at that stage (Li et al., 2006).

Nonetheless, in the current study patterns for all growth and development related genes were similar. Moreover, environmental parameters such as temperature influence these genes in embryos (Li et al., 2006; Nipkow et al., 2018) as well as larvae (Politis et al., 2017). Thus, these genes can be used as indicators to optimize rearing protocols for European eel embryos.

4.2.3. Genes related to lipid metabolism

The great importance of fatty acids for reproductive success and high egg quality is widely accepted and extensively studied (Sargent et al., 1999; Tocher, 2003), including in European eel (Støttrup et al., 2016). However, knowledge on the importance of maternal mRNA and the expression patterns of fatty acid metabolism genes throughout early development is scarce. In the present study, we investigated the expression dynamics of *cpt1a*, *cpt1b*, and *pigf5*, which are involved in β-oxidation. Here, though being maternally derived, we observed overall low mRNA levels of these genes during early development with strong increases after commencement of the embryos own transcription. Moreover, we found higher expression of cpt1b in the high and medium quality group compared to the low quality group. This is in agreement with a previous study on European eel, where a higher relative abundance of all three genes was found for the hatch group compared to the non-hatch group, but only during later embryonic development (Rozenfeld et al., 2016). Similarly, in Atlantic cod higher expressions of these genes were found in embryos originating from wild broodstock (high quality) compared to embryos obtained from farmed broodstock (low quality) (Lanes et al., 2013). In zebrafish, the knockdown of *cpt1a* lead to impaired lymphatic development demonstrating its importance for early development in fish (Zecchin et al., 2018). The expression pattern found in the current study may indicate a functional role in lipid metabolism for late embryonic development and possibly early larval development. In the orange-spotted grouper, *Epinephelus coioides*, *cpt1* expression was also initially low with a rapid increase during main organ formation processes, however, decreasing again towards hatch (Tang et al.,

2013). Interestingly, the expression of *cpt1* did not change over time in embryos and early larval stages of turbot, *Scophthalmus maximus* (Cunha et al., 2013), indicating the function and dynamics during early life history may be species specific.

4.2.4. Genes related to thyroid metabolism and signaling

Thyroid hormones (TH) play essential roles in growth, maturation, development and metabolism and have been extensively studied in humans, mammals and birds (Power et al., 2001). Knowledge on their function in early development in fish is still incomplete but THs are likely to be maternally deposited into the oocyte, regulating early development until the offspring are capable of endogenous hormone production (Brown et al., 2014). TH bind to nuclear thyroid hormone receptors (THR), which mediate their actions (Power et al., 2001). In European eel, four different subtypes of THR (thrαa, thrαb, thrβa, thrβb) and three different subtypes of deiodinases (*dio1*, *dio2*, *dio3*) have been characterized (Politis et al., 2018b). In the present study, most of the genes (except *dio1*) involved in thyroid metabolism and signaling showed relatively low initial mRNA levels but increased after the MZT and embryos own genome activation. Here, *thrαb*, *thrβa*, *thrβb* showed only slight increases towards hatch. A different pattern was found for *dio1* that appeared to have higher maternally derived levels during the early embryonic stages and more stable levels throughout embryogenesis, but with a slight decrease after MZT. Moreover, mRNA transcript levels in embryos of high and medium quality were higher compared to embryos with low developmental potential indicating that *dio1* may be of particular importance during early embryogenesis. In teleost, *dio1* and *dio2* have similar functions being capable to convert T4 (thyroxine) to T3 (3,5,30-triiodothyronine), while *dio3* is a purely inactivating enzyme (Orozco and Valverde-R, 2005). In zebrafish, knockdown of *dio1* and *dio2* had severe impacts on embryonic development (Walpita et al., 2010). Contradictory to our results, where *dio2* was hardly expressed during early stages, *dio2* was found to be of higher importance compared to *dio1* in zebrafish,

indicating that functional roles may be stage specific and vary among species. In the present study, the expression of *dio2* and *dio3* showed a pronounced peak towards hatch, which is in line with results from European eel larvae, where elevated levels of these two genes were observed in larvae at hatch (Politis et al., 2018b). Moreover, the presented study observed an association between E2 concentrations and *dio3* expression in the unfertilized egg as well as between T and *thrαb* indicating a possible interplay between the two hormone systems. Thyroid hormone receptors belong to the steroid-thyroid super family that also contains receptors for ligands, such as steroids, retinoids and vitamins (Power et al., 2001; Tsai and O'Malley, 1994) indicating cross-talk between the hormone systems through receptor binding (Duarte-Guterman et al., 2014). However, little is known about the extent of this in teleosts. In goldfish, injections with E2, T or 11-kt did not affect the expression of *thrαa*, *thrαb*, *thrβa* and *thrβb* in adult tissues (Nelson and Habibi, 2009). Nonetheless, in human cells a tissue specific positive effect of E2 on the expression of *dio3* was found (Kester et al., 2006). Overall, similar expression patterns of genes involved in thyroid metabolism throughout embryogenesis with increasing expression levels after MZT have been found for zebrafish (Campinho et al., 2010; Vergauwen et al., 2018), rainbow trout (Li et al., 2007), Atlantic salmon (Jones et al., 2002), sea bass (Nowell et al., 2001; Walpita et al., 2007) and fathead minnow, *Pimephales promelas* (Vergauwen et al., 2018). Previous results have shown the importance of thyroid metabolism on larval stages of Japanese (Kawakami et al., 2013) and European eel (Politis et al., 2018b, 2018c). Together, these findings may indicate that the thyroid hormone system is functional already during early stages of eel embryogenesis.

Altogether, results from this study deepen our understanding on the maternal transfer of steroid hormones and mRNA transcripts to eggs and their temporal changes throughout embryonic development in European eel. High levels of maternally derived DHP and E2 may influence developmental competence. As such, the ability of the embryo to metabolize their innate steroids

appears crucial to development, although the molecular mechanisms mediating these effects are not yet clear. Furthermore, the present study revealed pronounced changes in mRNA transcripts of genes related to growth, development, and metabolism during early ontogeny of the European eel.

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Table 1.

Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR.

Full name	Abbreviation	Function	Primer Sequence (5' 3') (F: Forward; R: Reverse)	Reference /Accession
Cyclin A2	*ccna2*	Reference	F: ATGGAGATAAAATGCAGGCCT R: AGCTTGCCTCTCAGAACAGA	
Cellular island	*cei*	Reference	F: CCTCAAACACCCCAACATCC R: AGCTCCTCCATGTACGTTGC	
Thyroid hormone receptor alpha a	*thαa*	Reference	F: GCAGTTCAACCTGGACGACT R: CCTGGCACTTCTCGATCTTC	(Politis et al., 2018b)
Insulin like growth factor receptor 1b	*igfr-1b*	Reference	F: ATGGGAATCTTCAGCTCTTTAGA R: TCAAACTCCTCCTCCAAGCT	
Heat Shock Protein 70	*hsp70*	Stress response	F: TCAACCCAGATGAAGCAGTG R: GCAGCAGATCCTGAACATTG	(Politis et al., 2018a)
Heat Shock Protein 90	hsp90	Stress response	F: ACCATTGCCAAGTCAGGAAC R: ACTGCTCATCGTCATTGTGC	(Politis et al., 2018a)
Growth hormone	*gh*	Somtaotropic axis	F: GGAGGAAGAGGAGCTGAAGA R: GGGGCAGGAAAATCACCATC	
Insulin like growth factor 1	*igf-1*	Somtaotropic axis	F: TTCCTCTTAGCTGGGCTTTG R: AGCACCAGAGAGAGGGTGTG	(Politis et al., 2018c)
Insulin like growth factor 2a	*igf-2a*	Somtaotropic axis	F: AGCCCAGAGGCTGAGGAG R: GATCAGATGTCGGTGGGATT	(Rozenfeld et al., 2016)
Insulin like growth factor 2b	*igf-2b*	Somtaotropic axis	F: CGGTCACAGAAGGGAATTGT R: GACGTCTCTCTCCGACTTGG	Rozenfeld et al., 2016)
carnitine O-palmitoyltransferase liver isoform-like 1a	*cpt1a*	Lipid metabolism	F: CCAGGCTGTGGATGAATCTT R: GCAAAGAGGACTGGAAGCTG	(Rozenfeld et al., 2016)
carnitine O-palmitoyltransferase liver isoform-like 1b	*cpt1b*	Lipid metabolism	F: TCTACGCTGGCTACGGAGTT R: ATAATGGGACTTCGCCCTCT	(Rozenfeld et al., 2016)
phosphatidylinositol glycan biosynthesis class F protein 5	*pigf5*	Lipid metabolism	F: ACAAGGTGTCCAAGGTCGTC R: GAAGGAGGACAGCAGGACAG	(Rozenfeld et al., 2016)
Deiodinase 1	*dio1*	Thyroid metabolism	F: AGCTTTGCCAGAACGACTGT R: TTCCAGAACTCTTCGCACCT	(Politis et al., 2018b)
Deiodinase 2	*dio2*	Thyroid metabolism	F: GAAGAGGAGGATCGCCTACC R: GCACTCTACCTCCGTCCAAA	(Politis et al., 2018b)

Deiodinase 3	*dio3*	Thyroid metabolism	F: TACGGGGCGTATTTTGAGAG R: GCTATAACCCTCCGGACCTC	(Politis et al., 2018b)
Thyroid Hormone Receptor alpha b	*thrαb*	Thyroid metabolism	F: GAAGCCTTCAGCGAGTTCAC R: ACAGCCTTTCAGGAGGATGA	(Politis et al., 2018b)
Thyroid Hormone Receptor beta a	*thrβa*	Thyroid metabolism	F: AGGAACCAATGCCAAGAATG R: GCCTGTTCTCCTCAATCAGC	(Politis et al., 2018b)
Thyroid Hormone Receptor beta b	*thrβb*	Thyroid metabolism	F: GAAGACTGAGCCCTGAGGTG R: AGGTAATGCAGCGGTAATGG	(Politis et al., 2018b)

Full name, abbreviation, function, and accession numbers or references for primers retrieved from previous studies are listed.

Table 2.

Data on quality parameters for reproductive success of female European eel, *Anguilla anguilla* and categorization into high, medium and low quality depending on developmental competence.

Female	Fertilization success (%)	Cleavage abnormalities (%)	Survival 48 hpf (%)	Hatch success (% fertilized eggs)	Quality group
1	3.63	n.d.	0.00	0.0	Low
2	12.50	39.15	3.84	13.7	Low
3	14.78	67.32	2.45	0.0	Low
4	37.65	38.79	17.04	2.6	Low
5	44.35	55.56	14.58	n.d.	Low
6	26.73	44.52	21.80	69.4	Medium
7	37.37	16.20	21.88	46.8	Medium
8	54.57	25.93	40.60	62.9	Medium
9	58.10	27.78	57.32	69.2	Medium
10	61.68	5.19	56.68	85.2	High
11	61.97	4.43	64.40	81.8	High
12	72.94	1.04	70.78	72.7	High
13	76.66	7.84	61.30	48.7	High
14	81.10	7.08	n.d.	90.2	High
15	89.41	2.24	81.75	87.7	High
16	91.49	6.57	80.82	78.5	High

n.d.: no data available

Table 3.

Steroid concentrations in European eel, *Anguilla anguilla*.

Steroid	Female plasma (ng/ml)			Unfertilized eggs (ng/g)			Embryos at 2 hpf (ng/g)		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
T	57.28 ±11.85	58.37 ±15.67	82.05 ±14.02	1.19 ±0.13	1.52 ±0.18	1.53 ±0.15	0.53 ±0.04	0.44 ±0.06	0.68 ±0.05
11-kt	8.56 ±2.17	11.59 ±2.88	7.75 ±2.57	0.47 ±0.09	0.46 ±0.12	0.59 ±0.11	0.17 ±0.08	0.17 ±0.10	0.22 ±0.09
cortisol	24.57 ±6.03	14.84 ±7.97	24.07 ±7.13	0.48 ±0.10	0.58 ±0.13	0.59 ±0.12	n.d.	n.d.	n.d.

T: testosterone; 11-kt: 11-ketotestosterone; hpf: hours post fertilization; n.d.: no data available

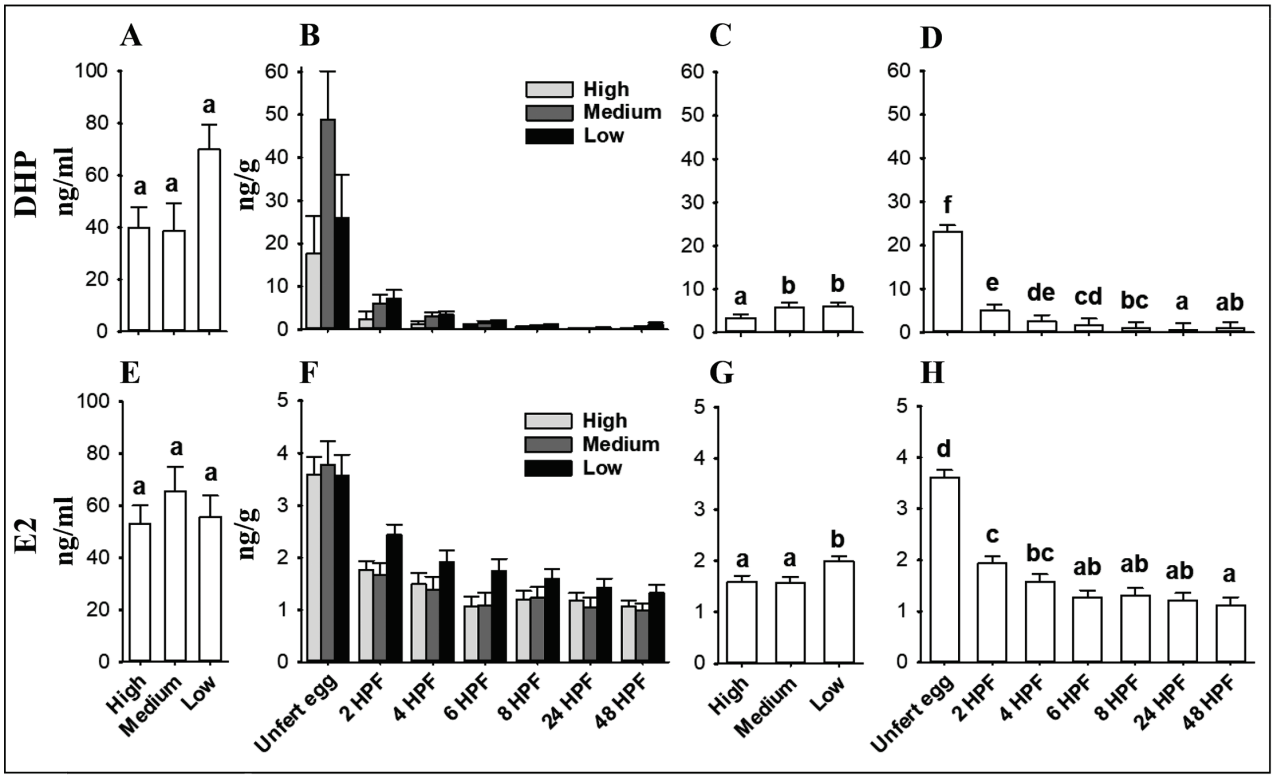

Fig 1. Steroid concentrations in European eel, *Anguilla anguilla*. (A) Post-stripping plasma DHP concentrations in female eels producing high, medium and low quality eggs, (B) DHP concentrations in eggs and embryos from high, medium and low quality groups over sampling time (C) DHP concentrations in different quality groups (main effect quality) and (D) E2 concentrations over sampling time (main effect age). (E) Post-stripping plasma E2 concentrations in female eels producing high, medium and low quality eggs, (F) E2 concentrations in eggs and embryos from high, medium and low quality groups over sampling time (G) E2 concentrations in different quality groups (main effect quality) and (H) E2 concentrations over sampling time (main effect age). Values represent means (± SEM) among females at each sampling time and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

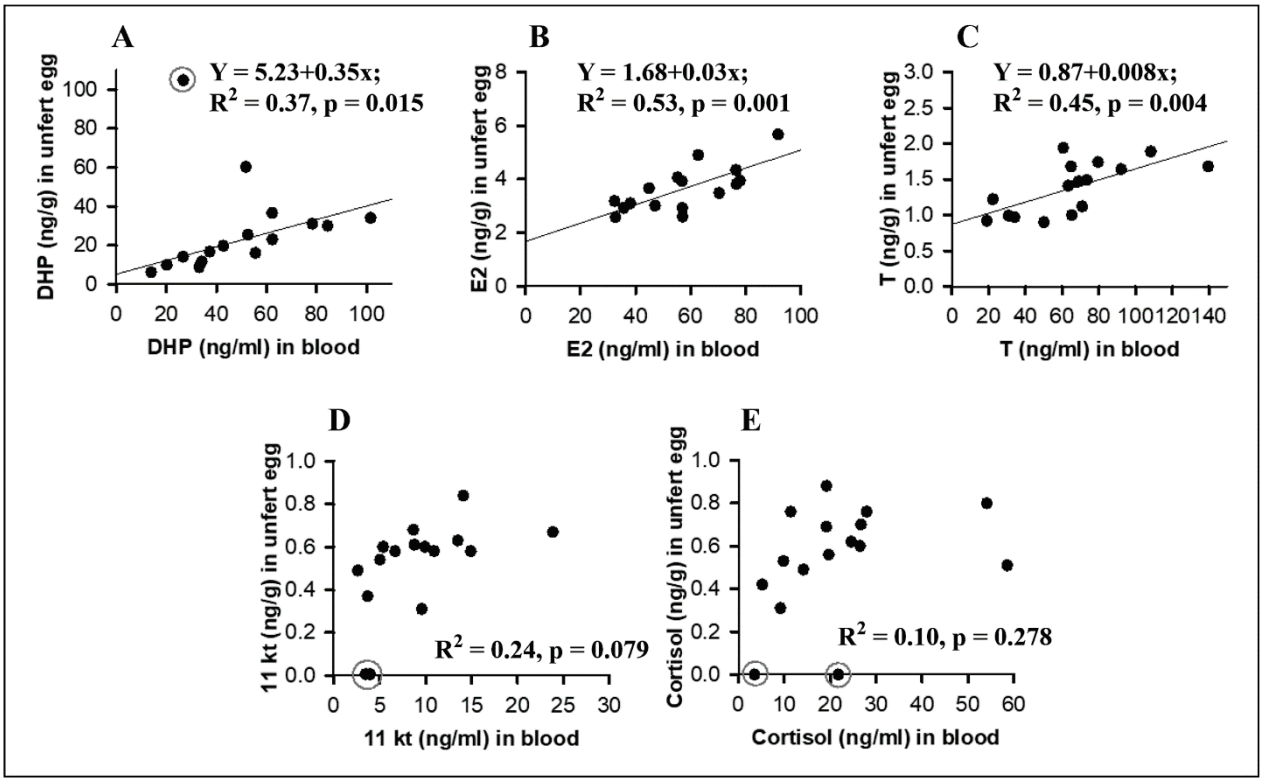

Fig 2. Association between female post-stripping steroid plasma concentrations and concentrations in the unfertilized eggs for (A) DHP, (B) E2, (C) T, (D) 11-kt, and (E) Cortisol. Circles mark outlier that have been removed from the regression analyses.

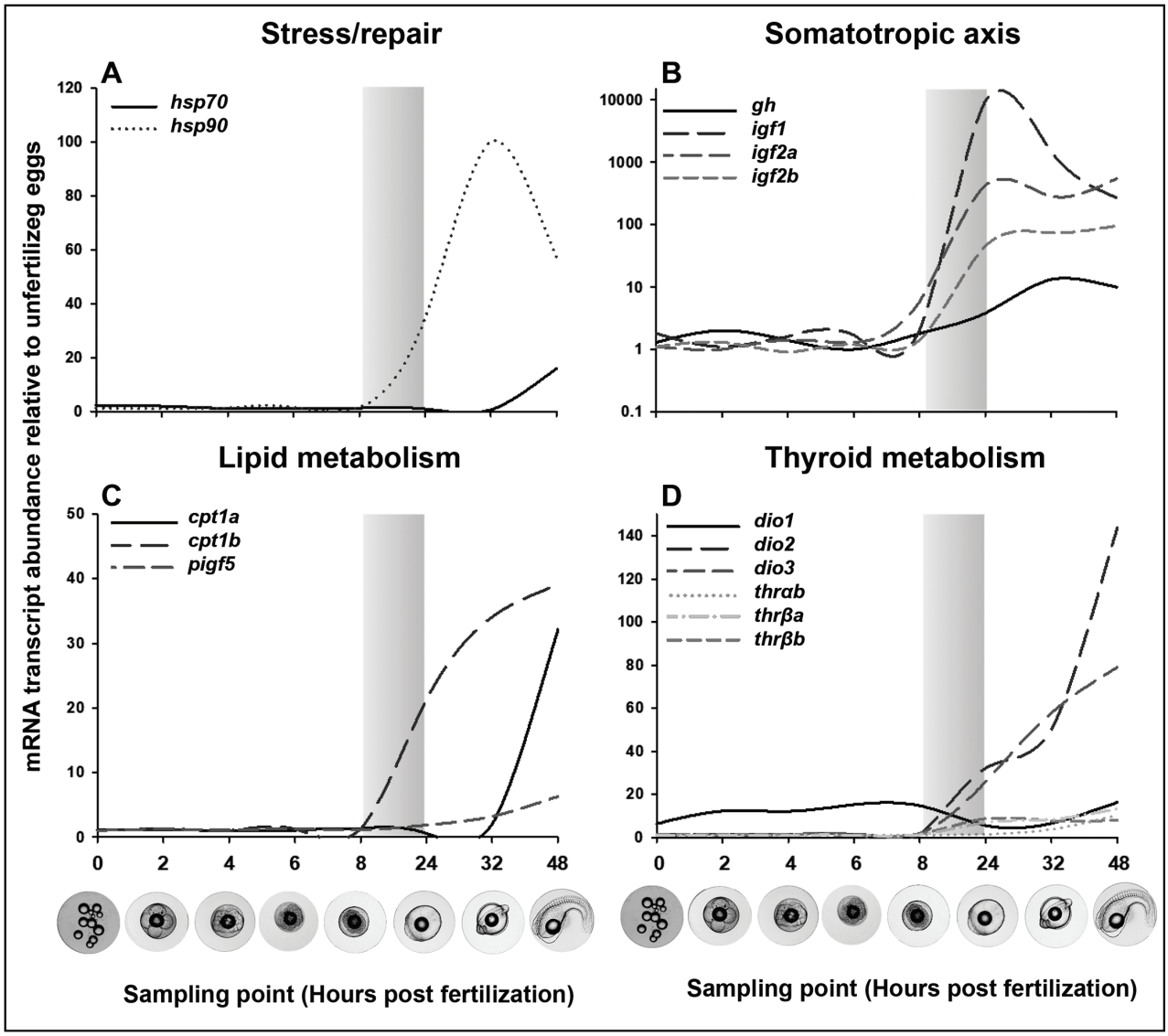

Fig 3. mRNA transcript abundance throughout embryonic development in European eel, *Anguilla anguilla*. Conceptual overview – mRNA abundance ($2^{-\Delta\Delta Ct}$) was calculated in relation to the average abundance in the unfertilized eggs of each gene. Relative mRNA abundance of (A) *hsp70, hsp90,* (B) *gh, igf1, igf2a, igf2b,* (C) *cpt1a, cpt1b, pigf5, and (D) dio1*, *dio2*, *dio3*, *thrαb*, *thrβa*, *thrβb*. Bar represents timeframe of maternal-to-zygotic transition (MZT).

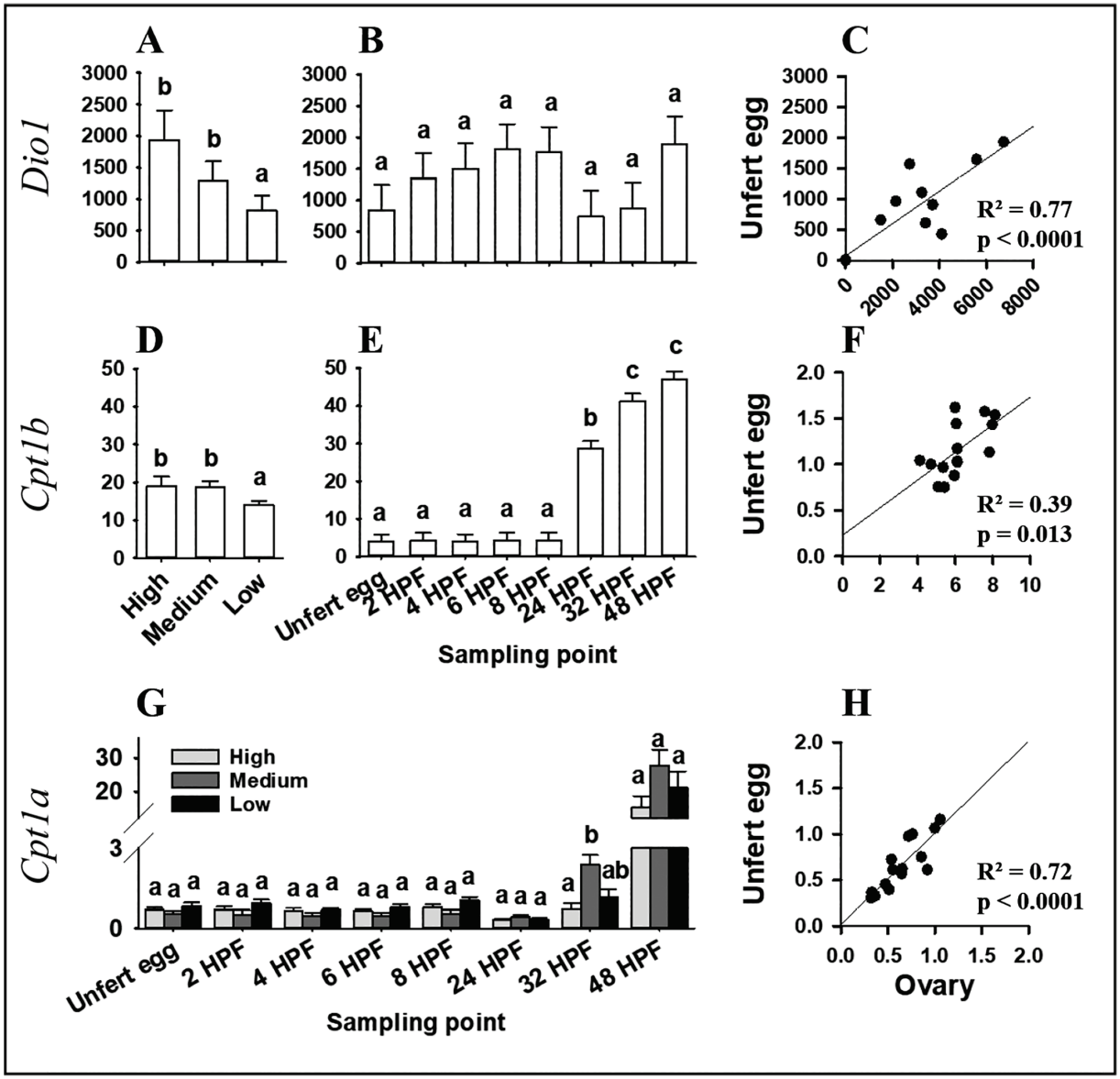

Fig 4. mRNA transcript abundance in European eel, *Anguilla anguilla*. Relative mRNA abundance of *dio1* (A) in different quality groups (B) throughout embryonic development and (C) association between *dio1* between mRNA abundance in female ovary and unfertilized egg, and relative mRNA abundance of *cpt1b* (D) in different quality group (E) throughout embryonic development and (F) association between *cpt1b* between mRNA abundance in female ovary and unfertilized egg, and (G) interaction of relative mRNA abundance of *cpt1a* between quality groups throughout development and (H) association between *cpt1a* between mRNA abundance in female ovary and unfertilized egg. Values represent means (± SEM) among females at each sampling time and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

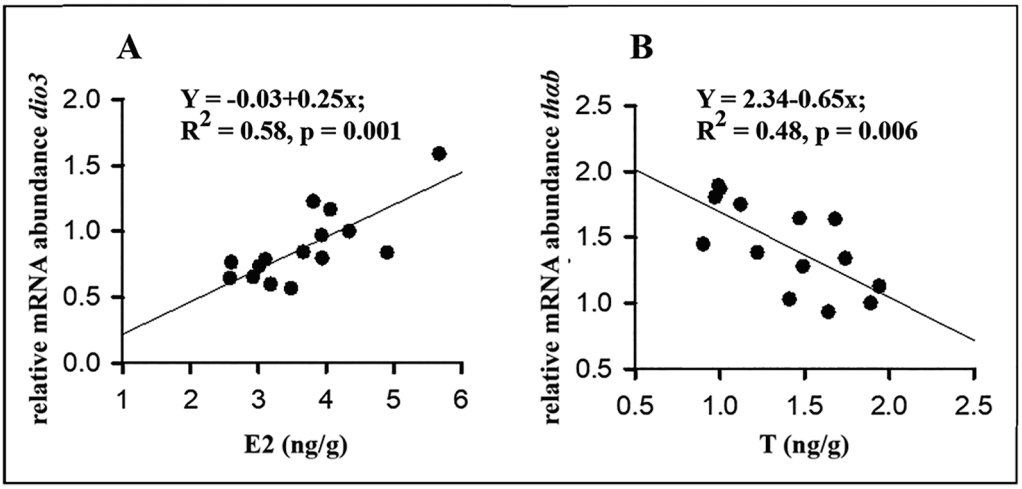

Fig 5. Relationship between steroid concentrations and mRNA abundance. Relationship in the unfertilized eggs between (A) E2 concentration and *dio3* mRNA abundance and (B) T concentration and *thrαb* mRNA abundance.

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