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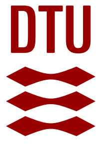

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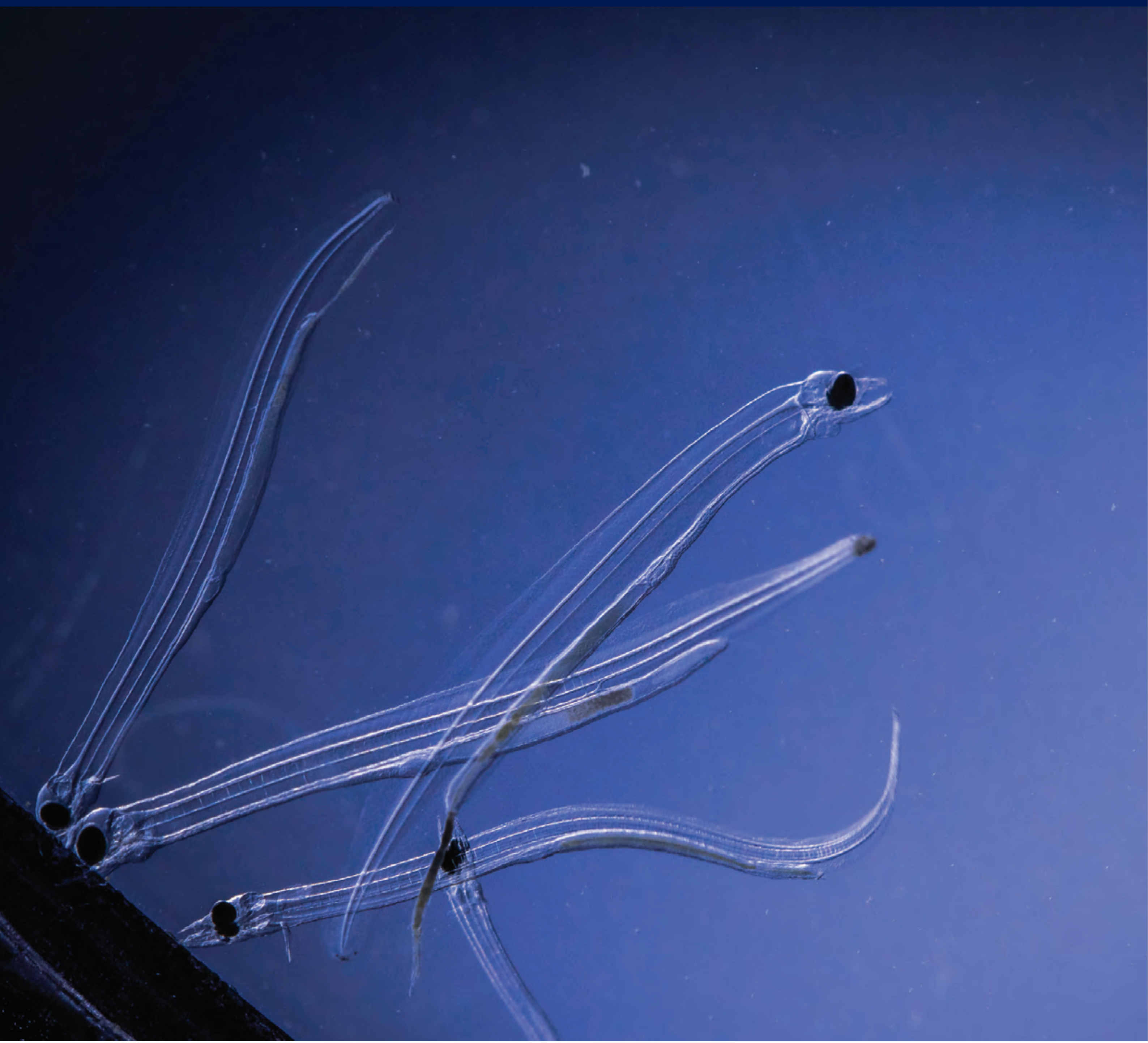
National Institute of Aquatic Resources

European eel larval quality and feeding regime: Establishing first-feeding culture

Elisa Benini

PhD Thesis



European eel larval quality and feeding regime:

Establishing first-feeding culture

PhD Thesis

Elisa Benini

Submitted: February 28, 2022
Kgs. Lyngby, Denmark

Technical University of Denmark
National Institute of Aquatic Resources
Section for Marine Living Resources

Report
2022

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Cover photo: European eel larvae feeding in Kreisel tanks.
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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.

The PhD studies were part of the project ”Improve Technology and Scale-up production of offspring for European eel aquaculture” (ITS-EEL) supported financially by the Innovation Fund Denmark, Grant no. 7076-00125B and ENV-“Fonden”. The PhD project, conducted between July 2018 and February 2022, was supervised by main supervisor Dr. Jonna Tomkiewicz and three co-supervisors; Dr. Sebastian N. Politis (DTU Aqua), Dr. Sofia Engrola (Centre of Marine Science, CCMAR, Portugal) and Prof. Dr. Anders Nielsen (DTU Aqua). Experimental work was carried out at the EEL-HATCH facility, located at DTU Aqua in Hirtshals, Denmark, while two external research stays took place at CCMAR in Faro, Portugal.

Prior to my enrollment as PhD student, I had valuable working experience at the EEL-HATCH facility, where I was employed as a Technical Assistant by Scandinavian Technical Marine Innovation (STMI), an industrial partner in the ITS-EEL consortium. 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 follow unanswered questions within eel larval culture and nutrition and I was privileged to qualify for a PhD fellowship.

During my PhD project, the spreading of Corona virus and the global pandemic forced me to change my planned work and adapt my studies to the new situation, including several lock-downs and general uncertainty. However, despite the challenges and associated delays, four studies were performed during this PhD project, each yielding a manuscript included in this thesis.

The studies were integrated in the ITS-EEL advancement of European eel first feeding larval culture, trying to fill the gaps of knowledge about factors affecting larval quality, nutrition and development.

In particular, the first study focused on offspring quality by analyses of the biochemical composition throughout development from fertilized egg to first feeding larvae. This study was important to substantiate insights on maternal influences on offspring quality, involving effects of assisted reproduction.

The three subsequent studies focused on generating new knowledge about influences of diets and dietary regimes on the development and functionality of the digestive and immune system as well as appetite, food intake, growth and stress response of the eel larvae during early development. Thus, Study 2 and 3 focused on the transition between

endogenous and exogenous stage by promoting the functional maturation of the gastro intestinal tract.

In this regards, Study 2 tested the potential benefit of gut priming agents (probiotic, prebiotic and the combination of the two – called synbiotics) to enhance the functional maturation of the digestive tract by steering the gut microbiota in yolk-sac larvae and exploring potential benefits on survival and development.

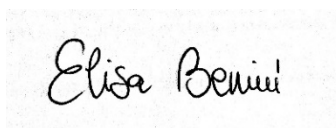
Successively, Study 3 focused on the benefit of introducing feed during the yolk sac stage, i.e. before the first feeding stage and earlier than the full digestive capacity is acquired. Here, the molecular expression of genes related to digestion, appetite, food intake, as well as stress and growth were analyzed throughout the first feeding window (between 8 and 18 dph), using three different diets and feeding regimes.

Thus, with Study 2 and 3, we aimed to evaluate the possibility to facilitate the transition from endogenous to exogenous feeding through stimulation of the functional gut development and the larval digestive capacity.

The last study, Study 4, focused on testing different diets in the quest to identify a suitable diet/feeding regime for first-feeding European eel larval culture. Through morphological and molecular analyses, we evidenced that one of the diets allowed successful transition between endo- to exogenous feeding. Although survival was limited, this is the first time that European eel larval food uptake has been sufficient to maintain their metabolism, while novel insights into the development of a functional digestive system will help advancing the establishment of a first feeding culture for this species.

Thus, the studies included in this thesis, overall aimed at filling gaps in knowledge about early life history stages of European eel under culture conditions, needed to establish a first-feeding culture. The knowledge gained within this project contributed to acquiring valuable information regarding eel larval digestion and development, providing one further step towards closing the life cycle of this critically endangered species in captivity.

Kgs. Lyngby, 28 February 2022



Elisa Benini

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English Summary

Development of closed-cycle hatchery technology is pivotal for sustainable aquaculture production of the European eel (*Anguilla anguilla*). In this context, the research conducted within this PhD project contributes to generating the knowledge needed to establish larval culture of European eel by addressing biochemical, nutritional and developmental factors influencing early larval stages. This involves experimental studies, which use an integrative set of tools to elucidate relations between offspring survival, developmental indicators and molecular mechanisms in the pursuit of insights into the early life history of the enigmatic eel larvae. The thesis frames the outcomes of four studies within three main topics: i) larval quality and biochemical composition, ii) maturation of the gut during the transition between endogenous and exogenous feeding and iii) first-feeding diets and dietary regimes for eel larval culture. All those aspects influence larval development and survival thereby being vital for advancing the establishment of feeding larval culture of European eel.

In aquaculture, variable egg quality from captive broodstock is a common problem affecting viable offspring production. A stable supply of high quality eggs requires comprehensive understanding of the factors that regulate gamete and offspring quality. For the European eel, reproductive protocols involve administration of pituitary extracts from carp (CPE) or salmon (SPE) to induce and sustain oocyte development and vitellogenesis. During vitellogenesis that lasts several months, yolk constituents (for example protein, lipid, and amino acid) are incorporated into the oocytes. In this regard, **Study 1** compared the effects of CPE and SPE on the biochemical composition of eggs and yolk sac larvae as well as the subsequent nutrient utilization by the developing larvae during the yolk-sac stage. The results showed that the type of pituitary extract used to induce vitellogenesis in female eel influenced the biochemical composition of the offspring, where SPE females produced more buoyant eggs with higher fertilization rate as well as larger larvae with more energy reserves, while CPE females spawned eggs with higher lipid and free amino acid content. Whereas these differences were marked at the egg, embryo and early larval stages, the composition of surviving larvae originating from the two treatments were similar by the end of the yolk sac stage. Overall, the yolk sac larvae utilized lipids and monounsaturated fatty acids as energy source, until they reached 8 days post hatch (dph), marking the time point, where larvae would benefit from the presence of exogenous nutritional stimulants, possibly such as gut-priming agents or prefeeding protocols, in order to meet subsequent energy and constituent requirements.

European eel larvae reach the first feeding stage around 10-12 dph at 18-20°C, which is characterized by attainment of a fully functional feeding apparatus as well as vision. However, molecular processes related to digestion and food intake are starting to be expressed already at 4 dph. At this point, introduction of gut priming agents or prefeeding may advance the transition from endogenous to exogenous feeding by stimulating digestive functions. Accordingly, in **Study 2**, commercially available gut priming agents

were tested, including prebiotics, probiotics and their combination, i.e. synbiotics, administered to the larvae during the endogenous feeding stage. Results revealed an increased mortality, when eel larvae were offered synbiotics, while both probiotics and synbiotics impaired somatic growth. Nevertheless, gene expression analyses indicated a prospective adaptive capacity towards earlier maturation of the larval gut, enhancing digestive capacity. The gut-priming agents and regimes applied in this experiment did not appear suited for eel larvae in culture, however, also water management strategies, when adding nutrients to larval culture, need attention e.g. avoiding overload of the recirculating system or the formation of debris on the tanks bottom, as microbial growth appears to interfere with larval survival.

In parallel, **Study 3** investigated the effect of early introduction of feed (prefeeding) on the transition from the yolk sac stage to active feeding larvae. Since a suited first feeding diet has not yet been developed for Euroepean eel larvae, three different feeds and feeding regimes were tested during the yolk sac stage and throughout the first feeding window. The results indicated that while prefeeding challenged larval survival, the early introduction of nutrients might benefit the functionality of the gastro-intestinal tract and equip the larvae with an improved digestive capacity. Although, none of the feeding regimes tested seemed to include a sufficiently balanced diet resulting in larval growth, one of the feeding regimes applied in this study resulted in the highest survival rates registered at that time point. In particular, the molecular results added insights of importance to the development of diets for first feeding culture of European eel larvae, as shown in one of the Feeding regimes, where gene expression together with survival data pointed towards a dietary composition, getting closer to the nutritional requirements of first-feeding European eel larvae.

In **Study 4**, three formulated diets were tested experimentally, providing the feeds to larval cultures from 10 dph, i.e. the onset of the first feeding window until 28 dph. The three diets, taking inspiration from larval feeds applied for Japanese eel (*A. japonica*), shared the same basic ingredients. Here, Diet 1 represented the simplest composition, while Diet 2 and 3 were modifications, where proteins with different molecular size were added. Thus, Diet 2 included fish hydrolysate with ~3 kDa protein size, while Diet 3 included whey protein of ~10 kDa. The results showed an early benefit (15 dph) of Diet 1 in terms of initially improved larval survival, morphological development and gene expression. However, at 22 dph, larvae fed Diet 3 showed upregulated expression patterns of the genes encoding the major digestive enzymes, a downregulation of *ghrelin*, encoding the “hunger hormone”, together with an improved survival and growth. Moreover, the expression of digestion, food intake and growth related genes continued to increase towards 28 dph for larvae fed Diet 3. Thus, it seems that the inclusion of more complex dietary proteins (as in Diet 3), but not hydrolyzed peptides (as in Diet 2) is beneficial in order to promote larval ontogenetic performances after the successful transition to exogenous feeding. In spite that survival was limited, larvae fed Diet 3 survived throughout and for the first time, beyond the first feeding stage, providing important information in relation to the nutritional requirements of first feeding larvae of

this species. The results, therefore, provide a useful basis for future research efforts towards establishment of larval culture of European eel.

Altogether, the combination of methods applied in this PhD project has provided novel insights into the biochemistry, resource utilization, developmental physiology and nutritional requirements of European eel larvae in culture. Still, the establishment of a first feeding culture of European eel is at a pioneering state. In the present studies, based on consecutive targeted experiments, larval survival and growth has been incrementally improved and insights substantiated. It is the first time that successfully feeding leptocephalus larvae survived beyond the first feeding stage enabling a morphological and molecular characterization of development. These results pave the way for forthcoming extended feeding experiments together with integrating multi- and interdisciplinary research tools that will benefit hatchery development and conservation of the critically endangered European eel.

Dansk Resume

Bæredygtig akvakultur af den europæiske ål (*Anguilla anguilla*) kræver udvikling af klækkeriteknologi til produktion af glasål i en lukket cyklus. Forskningen, der er gennemført i dette ph.d.-projekt, bidrager i denne sammenhæng til at skabe den viden, der er nødvendig for at etablere larvekultur af denne art. Fokus er på biokemiske, ernæringsmæssige og udviklingsmæssige faktorer, der påvirker de tidlige livsstadier, hvor eksperimentelle studier integrerer forskellige analysemetoder for derigennem at belyse sammenhænge mellem ynglens overlevelse, udvikling og fysiologi. Formålet er derigennem at øge indsigten i den tidlige livshistorie, som er stort set ukendt for ålelarver i naturen. Afhandlingen omfatter resultaterne af fire studier, som falder inden for tre hovedemner: i) larvekvalitet og biokemisk sammensætning, ii) modning af fordøjelsessystemet ved overgangen mellem endogen og eksogen fødeoptagelse og iii) startfoder og fodringsregimer i larvekultur. Alle disse aspekter påvirker larvernes udvikling og overlevelse og er derved afgørende for etableringen af larvekultur for den europæiske ål.

Svingende kvalitet af æg fra stamfisk i opdræt er et almindeligt forekommende problem, der påvirker produktionen af levedygtigt afkom i akvakultur. Grundlæggende kræver en stabil produktion af højkvalitet afkom kræver en indgående forståelse af de faktorer, der påvirker forplantningen og kvaliteten af afkommet. En komplikation relation til ål er, at forplantning involverer assisteret reproduktionsteknologi, hvor hormonbehandling i form af hypofyseekstrakter fra karpe (CPE) eller laks (SPE) anvendes som middel til at igangsætte og opretholde udviklingen af ægceller. Det omfatter vitellogenesen, hvorunder blommemasse, dvs. proteiner, lipider, aminosyrer, indlejres i ægcellerne over en periode på flere måneder. I denne sammenhæng har Studie 1 undersøgt, hvordan CPE og SPE, påvirker den biokemiske sammensætning af æg og blommesæklarver, samt hvordan larverne udnytter næringsstofferne i blommesækken gennem deres udvikling under blommesækstadiet. Resultaterne viste, at de to typer hypofyseekstrakt påvirkede den biokemiske sammensætning af afkommet forskelligt. Således producerede de SPE-behandlede hunål flere befrugtede æg og større larver med flere energireserver, mens CPE-behandlede hunål producerede æg med et højere indhold af lipid og frie aminosyrer. Mens disse forskelle var markante for de ubefrugtede æg, æg med fostre og nyklækkede larver, var sammensætningen af larverne fra de to behandlinger ens ved slutningen af blommesækkestadiet. Overordnet brugte larverne lipider og monoumættet fedtsyre som energikilde gennem blommesækfasen fra klækning til dag 8, som nærmer sig det tidspunkt, hvor larverne skal begynde til tage næring til sig udefra for at opfylde energi og konstituerende krav.

Larver af den europæiske ål når det fødesøgende stadium omkring 10-12 dage efter klækning ved en temperatur på 18-20°C. Stadiet er kendetegnet ved et fuldt funktionelt fordøjelsessystem og udviklet syn. De molekylære processer, der er relateret til fordøjelse og fødeindtagelse er imidlertid allerede sporbare 4 dage efter klækning. På dette tidspunkt kan introduktion af fordøjelsesfremmende midler eller næringsstoffer

muligvis fremme overgangen fra endogen til eksogen fødeoptagelse ved at stimulere fordøjelsesfunktionerne. Studie 2 testede således effekten af kommercielt tilgængelige præparater inklusiv tilførsel af et præbiotisk middel, et probiotisk og kombinationen af begge (synbiotika) til kultur af blommesæklarver. Resultaterne viste imidlertid en øget dødelighed ved tilsætning af synbiotika og samtidig nedsatte både probiotika og synbiotika den somatiske tilvækst. Ikke desto mindre indikerede genekspressionsanalyser en adaptiv udvikling mod tidligere modning af tarmsystemet, hvilket kan forbedrede fordøjelseskapaciteten. De fordøjelsesfremmende midler og tilsætnings-metoden, der blev anvendt i dette eksperiment, så ikke ud til at være egnede i kultur ålelarver, men forsøget indikerede samtidig, at udviklingen af effektive vandbehandlingssystemer i samspil med næringstilførsel kræver opmærksomhed, da mikrobiel vækst synes at påvirke larvernes overlevelse.

Parallelt med det foregående studie undersøgte Studie 3 tilsvarende effekten af tilsætning af foder tidligt i blommesækkestadiet. Da der endnu ikke er udviklet et egnet startfoder til kultur af ålelarver, blev tre forskellige foder- og fodringsregimer testet. Selvom larveoverlevelsen blev reduceret gennem den tidlige introduktion af foder, viste resultaterne, at den tidlige introduktion af næringsstoffer kan gavne fordøjelsesfunktionen og muligvis udruste larverne med en forbedret fordøjelseskapacitet. Selvom ingen af de testede fodertyper så ud til at indeholde den rette kombination af næringsstoffer, og larveren dermed ikke viste tegn på vækst, resulterede en af fodertyperne i de højeste overlevelsesrater registreret på daværende tidspunkt. Her tilføjede de molekylære resultater ny viden om udviklingen af fordøjelsessystemet, hvilket har betydning for udviklingen af foder til larvekultur.

I Studie 4 blev tre specialdesignede diæter testet eksperimentelt. Her blev foderet tilsat kultursystemerne fra og med, at larverne var 10 dage gamle til de var 28 dage. De tre diæter, inspireret af larvefoder udviklet til japansk ål *(A. japonica*), delte de samme grundlæggende ingredienser. Diæt 1 repræsenterede den enkleste sammensætning, mens Diæt 2 og 3 var modifikationer med tilsætning af proteiner med forskellig molekylstørrelse. Diæt 2 inkluderede således fiskehydrolysat med en proteinstørrelse på ca. 3kDa, mens Diæt 3 inkluderede valleprotein med en størrelse på ca. 10 kDa. Fodring med Diæt 1 gav de bedste resultater tidligt i forløbet (15 dage efter klækning) i form af forbedret larveoverlevelse, udvikling og genekspression. Efter 22 dage viste larver fodret med Diæt 3 imidlertid en opreguleret ekspression af gener, der koder for de vigtigste fordøjelsesenzymer, en nedregulering af "sulthormonet" *ghrelin* sammen med en forbedret overlevelse og vækst. Ekspressionen af gener relateret til fordøjelse, fødeindtagelse og vækst fortsatte med at stige mod dag 28 for larver fodret med Diæt 3. Det ser således ud til, at inklusion af mere komplekse proteiner (som i Diæt 3), men ikke hydrolyserede peptider (som i Diæt) 2) fremmer larvernes ontogenetiske udvikling efter en overgangen til eksogen fodring. Larver fodret med Diæt 3 klarede overgangen til eksogen fødeoptagelse bedre end de øvrige og var i stand til at opretholde livsnødvendige funktioner og udvikle sig efter blommesæk-stadiet. Studiet gav derigennem vigtige oplysninger i forhold til ernæringsbehovet i den tidlige larvefase og

har skabt et nyttigt grundlag for den efterfølgende forskningsindsats til etablering af larvekultur for denne art.

Etableringen af larvekultur af europæisk ål og udviklingen af foder er stadig i en pionerfase. Her har kombinationen af metoder anvendt i dette PhD-projekt bragt ny viden om larvernes biokemi, ressourceudnyttelse, fysiologi og ernæringsmæssige behov i kultur. Gennem de fire studier, baseret på en række målrettede eksperimenter, er larvernes overlevelse og vækst gradvist blevet forbedret, og indsigten i vigtige processer styrket. Det er således første gang, at der er lykkedes at opnå larver, som overgik til og etablerede sig i det første fodringsstadie, hvilket har muliggjort en morfologisk og molekylær karakterisering af udviklingen mod leptocephalus-stadiet. Disse resultater baner vejen for kommende scenarier med udvidede fodringseksperimenter som sammen med integration af tværfaglig forskning kan lede til udvikling af klækkeriteknologi og bidrage til bevarelse af den kritisk truede europæiske ål.

Synthesis

1. The fish larvae

1.1 Factors affecting larval survival in nature

Among teleost fishes, a remarkably wide range of biological adaptations has evolved. Fish have adapted to live in extreme conditions, from the deep sea, in the cold Antarctic, or in warm fresh waters of high alkalinity and low oxygen (Chamber and Trippel, 2012). Along with these adaptations, fish show an impressive array of morphological, physiological and behavioral specializations. Despite difference in biology and ecology between species, the future of the population is shaped by the rate of reproduction, growth and survival (Rodhouse et al., 2014.; Govoni, 2005). Therefore, studying these factors and their interconnections is not only interesting from an academic point of view but essential to sustainable exploitation and management of fish stocks as source of food for the human race.

Knowledge about the reproductive cycle of a species is imperative to sustainable management and aquaculture. In particular, teleosts exhibit a variety of reproductive strategies, shaped by adaptations to the environment over the past 500 million years. These strategies are essential for the survival of the species and allow fine-tuning of the timing of reproduction with the seasonal environmental changes. Indeed, most temperate teleosts are seasonal spawners with the release of gametes programmed so that the progeny is produced when conditions are most favorable (Cabrita et al., 2008). This involves a range of environmental (light, temperature, salinity), nutritional (feed quality, quantity) and social (sex ratio, size structure, dominance/hierarchy) factors that play important roles in the synchronization of the reproductive act and successful offspring production. Differences between fish existing species in terms of seasonality of reproduction, fecundity or spawning type are, to a large extent, the consequence of adaptation to particular photic and thermal niches into which groups have been pushed by a variety of unrelated selection pressures (Mylonas et al., 2010; Migaud et al., 2013).

Moreover, fish have a very high growth potential and major morphological and physiological transformations can happened during their life. This is especially true during the early development (among others: Hjort 1914; Kamler 1992) when newly hatched larvae quickly develop throughout the yolk-sac stage (Holt, 2011). By the end of this stage, the larvae need to shift from endogenous to exogenous feeding. Thus, larvae of oviparous fishes hatch with a yolk sac, which contained the nutrients necessary to grow and survive for a limited amount of time. When the yolk sac is exhausted, larvae need to be able to feed autonomously as well as digest and assimilate food items. In this regard, high mortality rates generally are experienced during the larval stage, resulting in high variabilities in larval recruitment (Hjort 1926; Houde 2008). Nevertheless, the lack of a suitable food items in the water column matching the larval mouth size and auxiliary

feeding apparatus (e.g., branchial and jaw elements) is among the most recognized factors of low survival rate (Garcia et al.,2020). Intrinsically, fish larvae have a window of opportunity to start feeding (first-feeding window) and if they do not start ingesting and digesting the food during that window, they will go through irreversible starvation and perish (Blaxter, 1974). For these reasons, the larval stage has been labelled as the “critical period” in the life of a fish as high mortality during larval stage can have a deep impact on year-class strength (and recruitment) in marine fishes (”Critical period” hypothesis by Hjort, 1926).

Moreover, larval mortality is related to the match between fish spawning, larval production and larval prey availability (i.e. zooplankton bloom). As such, the “Match-Mismatch hypothesis” emphasize the importance of the presence at the same time in the same place of fish larvae and their prey (Cushing, 1990). In this regard, the environmental conditions can deeply affect the recruitment of fish larvae. Thus, the “Stable Ocean” hypothesis by Lasker (1981) and the “Optimal environmental window” by Cury and Roy (1989) suggest that environmental factors as wind and associated micro-turbulences can induce temporary vertical stratification of the water column creating aggregations of fish larvae and prey in the same water layer, which support high feeding, survival, and recruitment.

Another important factor affecting survival during the larval stage is predation (Bailey and Houde, 1989). The risk of death due to predation changes dramatically during the first few days after hatching, probably due to the rapid increase in length and/or changes in behaviour related to development during this period (Warkentin, 1995). The view most commonly expressed in the literature is that larger and older larvae are less vulnerable to predation, hence fast growth improves survival potential (“Big is better” hypothesis by Litvak and Leggett, 1992). In fact, larger larvae have stronger swimming capabilities than smaller ones and fast-growing larvae experience a shorter duration of the larval period, decreasing the period of time, when they are most vulnerable to predators (Pepin and Myers, 1991).

The ability of larvae to grow faster depends not only on the diet, but also on the parental effects and on environmental oscillations (Chambers and Leggett, 1996). Environmental factors, which include the physical and biotic processes acting on developing eggs and larvae such as temperature, salinity, turbidity and water turbulence, explain less than 40% of variation in larval growth (Wilson and Meekan 2002; Caldarone, 2005). This suggests that other processes, such as parental effects, must account for a substantial amount of variability in growth. Maternal contributions are generally considered more important than paternal contributions due to nutritional provisioning of the embryo (Bernardo, 1996). In oviparous fishes, embryonic and larval characteristics such as egg size, developmental rate, metabolism, growth and viability are affected by the body condition and genotype of the female parent (Chambers, 1997; Chambers and Leggett, 1996; Green and McCormick, 2005). In particular, young and small females in many stocks produce eggs and larvae that are smaller and inferior to those from older, larger females. There is evidence that such effects can reduce potential for survival of early life

stages and recruitment success (Trippel et al., 1997; Rideout et al., 2004). The non-genetic maternal contributions take many forms that directly influence survival probabilities of larvae, including nutritional reserves (Gagliano and McCormick 2007), levels of developmental and metabolic hormones, mRNA transcripts, etc. (Brown et al. 1989; McCormick 1998;1999), and parental care (Bernardo, 1996).

Therefore, high mortality rate during early life history of fish is to be expected in natural populations relating to the nutritional status of the larvae, (presence of prey), the presence of predators as well as environmental and parental factors. Still knowledge about factors affecting larval survival and recruitment and data are incomplete and primarily available for an array of economically important species.

1.2 Factor affecting larval survival in aquaculture

Aquaculture is the fastest growing sector for food production with an estimated 88 % of the total fish production being used directly for human consumption. The total number of cultured aquatic species worldwide has amplified by 26.7 % over the past years and now almost 600 species of aquatic organisms are being cultured (Tacon, 2020). Worldwide, the contribution of aquaculture to total seafood production has increased to 46.8 %. In Europe, however, the aquaculture share only accounts for 18 % illustrating a potentially unexploited market share to comply with the increasing demand for seafood production (Tacon, 2020).

The dynamic development and growing impact of aquaculture on the economy hinge on its sustainability. Here, 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). The main advantages of RAS technology is not only the reduced use of water, but also manageable rearing environments and constant biophysical conditions (Dalsgaard et al., 2013; Ebeling and Timmons, 2012). Thus, due to stable environmental conditions, regulated food supply and absence of predators, the survival rate for fish, and especially during the larval stage, is generally higher under aquaculture conditions than in nature.

However, one of the big constraints in aquaculture production is the mortality rate during early life history and high quality variability between batches. In this regard, it has been estimated that, only the 5-10% of eggs of the most common cultured marine fish species (seabass, seabream, halibut and turbot) reach the juvenile stage (Migaud et al., 2013). This is partially due to the underdeveloped form at which fish larvae hatch, with sometimes extreme physiological and morphological changes occurring during the embryonic and larval stages as well as lack of knowledge about the requirements during this critical period (Govoni, 2005). Moreover, larval developmental physiology and nutritional requirements may vary among species, even within the same family. Consequently, many specific processes cannot directly be extrapolated from findings obtained in model species and require specific studies. Knowledge about the ecology,

physiology and morphology of fish larvae is an absolute necessity for the development of a stable aquaculture production and therefore strongly encouraged.

1.2.1 Egg and larval quality

In aquaculture, high variability in eggs quality is a major issue and deeply influence the success of the production. The quality of eggs is defined as the ability of be fertilized and develop into a normal embryo (Bobe and Labbé, 2010). However, a broader definition includes "*the quality of an egg is determined by the intrinsic properties of the egg itself, by its genes, and by the maternal mRNA transcripts and nutrients contained within the yolk, all of which are provided by the mother*" (Brooks, 1997). Therefore, it is important to develop methods to evaluate egg quality. Among those, parameters as buoyancy or pigmentation of the egg are simple tools used to identify non-viable eggs. As such, buoyancy of pelagic eggs is often better in egg batches that develop normally as shown in Atlantic cod *Gadus morhua* (Kjesbu et al., 1992), Japanese eel *Anguilla japonica* (Unuma et al., 2005) and other species (Kjørsvik, 1994). Besides, in species that produce transparent eggs, the shape of the first embryonic cells (blastomeres) and the patterns of cell division can be assessed to identify abnormal development during early embryogenesis (Shields et al., 1997; Keller et al., 2008; Rideout et al., 2004). With this in mind, survival at a specific embryonic stage is one of the most common and relevant ways of characterizing the ability of the fertilized egg to develop successfully.

Another way that egg quality can be investigated is through the determination of its composition. Knowledge about the biochemical composition of eggs are very valuable as, after fertilization, the oviparous fish eggs operate as closed systems with only insignificant amounts of solutes and water exchanged freely, as a result of an extremely low permeability of the egg surface membrane (Kamler, 2008). In addition, the main component of eggs is yolk that represents the source of energy and materials for developing embryos and yolk-sac larvae, and the amount and composition of yolk are pivotal for the successful embryonic and post-embryonic development (Kamler, 1992). A vast amount of data, including numerous fish species, has been assembled regarding the biochemical composition and the patterns of nutrient allocation during the yolk-sac stage of larval development (Kamler, 2008). Despite major difference between taxa, it is possible to identify a certain pattern. Before hatch, embryos goes through with a short period of carbohydrate use, before switching to free amino acids (FAA) as the most important fuel. After hatching, lipid utilisation follows in response to the increased energy request. Thereafter, close to the first feeding stage, remaining lipids provides energy for swimming and different activities. When no more reserves are available for the larva (end of the endogenous stage), body protein-bound amino acids are mobilised (e.g. vitellogenin degradation) creating a second peak of amino acid catabolism. However, the timing and extent of these shifts is species-specific and modified by egg properties (with or without oil globule) (Kamler, 2008; Rønnestad et al., 1999).

1.2.2 *Offspring quality and broodstock*

The evolution from a population-based, single factor approach to a multi-disciplinary approach, unrevealing differences between organisms within and between populations has identified several environmental and biotic factors as important sources of variability in offspring quality. Moreover, factors as the female age and size or the male sperm motility as well as the compatibility between parents have been proven to deeply influence the offspring quality and performance (Kamler, 2005). The concept that larger, older or better condition females are producing offspring of better quality has been around since the 1960s (Nikolskii, 1962). Thereafter, this theory has gaining increasing support through laboratory and field experiments (Bergenius et al., 2002; Meekan and Fortier, 1996; Wright and Gibb, 2005). However, the link between variation in larval body size at hatching and recruitment strength is still largely theoretical (Green, 2008).

In aquaculture, the environmental conditions for rearing broodstock can be controlled and optimized (constant, temperature, salinity, food availability, no predators, among others), however, often aquaculture practices experience offspring quality problems. Such problems may relate to suboptimal temperature, photoperiod, nutrition etc. In some species, the reproductive cycle (i.e., gonadal development, leading to gamete production) needs to be triggered by the occurrence of a specific range of temperatures (Brown et al., 2006; Migaud et al., 2013). Moreover, exposure of female broodstock to suboptimal water temperatures during reproductive development may lead to reduced offspring quality. For instance, exposure of female rainbow trout *Oncorhynchus mykiss* to 17°C for a few weeks before ovulation leads to reduced embryonic survival, increased malformation rate, and increased occurrence of triploid fry (Aegerter and Jalabert, 2004).

The reproductive cycle is also very sensitive to photoperiodic signals. In many species e.g. in temperate and subpolar climates, sexual maturation is triggered by changes in photoperiod (Norberg et al., 2001) alone or in combination with temperature (García-López et al., 2006; Bromage et al., 2001). This strong relation between reproductive cycle and photoperiod is benefiting aquaculture, allowing to obtain out-of-season breeding or egg production by manipulating light conditions. However, such techniques can cause egg quality problems, even though the importance may vary depending on the species, the type of artificial photo-thermal regime, and the physiological status of broodstock when treatment is applied (Bromage et al., 1992; Bobe and Labbé, 2010; Migaud et al., 2013).

Furthermore, broodstock nutrition can directly influence egg yolk composition and therefore affect embryos and yolk-sac larval quality, since embryos will subsequently utilize egg yolk until first feeding. It is therefore important that broodstock diets are optimized nutrients including also vitamins and minerals besides fatty acids and amino acids to ensure good larval survival and early development (Izquierdo et al., 2001). Thus, broodstock diets should be formulated to ensure that all essential nutrient requirements are met for the species being cultured (Migaud et al., 2013). The lack of

key components (e.g., vitamins) in the diet or suboptimal feeding levels can lead to major adversities in egg quality (Brooks, 1997).

Together with nutrition, broodstock management techniques can induce egg quality problems. The overall impact of suboptimal conditions on egg quality is highly dependent on the species, the techniques used, and the physiological status of the fish, including stress level. Interestingly, it was shown that environmental factors and husbandry practices can result in specific egg quality problems such as specific malformations (Bonnet et al, 2007). Similarly, differences exist in egg composition between populations exhibiting different domestication levels, which could be linked to differential egg quality (Bobe, 2015; Nynca et al., 2020). However, for some species, recreating the natural environment is difficult and unable to meet the requirements, increasing the level of stress and failing to reproduce naturally. When natural spawning does not occur, artificial fertilization protocols to induce the production and stripping of the gametes are applied (e.g. Mylonas et al., 2010). In this regards, for some species, females are able to go through ovulation but fail in the spawning event, while for other species gamete development simply does not occur. In all these cases, the gametes must be stripped and fertilization performed artificially with ovulation in the latter case being induced using hormonal therapies. The impact of hormonal induction on gamete quality remains poorly described in most species and sometimes is limited to embryonic survival in the early stages (Mylonas et al., 2010).

All together, these factors indicate that the broodstock rearing techniques and environmental conditions potentially can have a major impact on the quality of the offspring. This also applies to application of assisted reproduction under aquaculture conditions, where refinement of protocols is extremely important.

1.2.3 Environmental and rearing conditions for larval culture

Once the egg is released into the water column, its development is dictated by the reserves included in the yolk sac in combination with the environmental conditions available. In aquaculture as in nature, the environmental condition plays a vital role in the development of the organism. For instance, water temperature during embryonic incubation and the yolk-sac stage is particularly important as it directly affects metabolism, development, and subsequently survival of the progeny (Blaxter, 1991). Thus, suboptimal temperatures can significantly affect egg mortality at early stages of development (Brooks, 1997).

Similarly, the effects of variations in salinity and oxygen content on larval fish can be deleterious. Salinity affects the osmoregulation, energy budget, feeding, growth, and development of fish (Holliday ,1969; Przeslawski et al., 2015; Tandler et al., 1995; Bœuf and Payan, 2001). In nature, drastically changes in salinity may happen as consequence of extreme weather events (e.g., floods and storms) profoundly affect embryo incubation and larval growth (Manuel et al., 2021; Gong et al., 2018). In captive breeding of marine species, salinity is prone to fluctuations, e.g. due to evaporation increasing salinity. Hatch success and malformation rates have been related to the salinity range. Thus, Shi et al.

(2010) reported that the optimal hatching and lowest malformation rates of pomfret *Pampus punctatissimus* embryos occurred at a salinity range from 29 and 32 psu. Similarly, low levels of dissolved oxygen in the water column are considered to be primary stressors in fish, a situation that is particularly important for early life stages, where oxygen depletion is likely to affect survival and the success of larval development (Breitburg et al., 1999; Cadiz et al., 2018). When hypoxia occurs at early life stage, detrimental effects on survival and growth of developing larvae has been observed (Pörtner and Peck, 2010). Therefore, understanding the specie-specific demands of offspring to for example temperature, salinity, and oxygen on larval performance is essential for the development of an effective hatchery.

Additionally, light is also a key environmental factor, which is dictating and synchronize all life-stages of fish, from embryo development to sexual maturation (Migaud et al., 2010) Light influence not only the vision and therefore the capacity of foraging and catching prey, but also affect pineal organs and brain photoreceptors, influencing development during the larval stage (Villamizar et al., 2011). Moreover, depending on the organisms phototaxis, larvae may be attracted to the surface of the water under high light intensities (positive phototaxis) or avoid light (negative phototaxis) and specific light spectra compared to low light intensities or dim light. Thus, light conditions is a key factor for fish larvae development and hatchery light conditions need to be adapted to the species requirements.

Moreover, offspring survival is influenced by the rearing conditions and methods used for incubation of the embryos and culture of the newly hatched larvae, which have significant power on the fate of the offspring (Nielsen et al., 2007; Salilew-Wondim et al., 2014). For example, optimized tank design and water hydrodynamics can provide uniformity of rearing conditions (Gorle et al., 2020), fast elimination of waste products (non-ingested feed and faeces)(Cripps and Bergheim, 2000) and uniform distribution of larvae (Dauda et al., 2019; Ebeling and Timmons, 2012). A suitable rearing system and specie-specific and stage-specific tank design are important to maintain high water quality, help in the distribution of the food and reduce the possibility of opportunistic bacteria to bloom.

Therefore, it is of primary importance for the establishment of larva culture to identify the optimal ranges of environmental conditions and suited rearing systems and techniques.

1.2.4 Larval feeding and nutritional requirements

A digestive system and a highly specialized feeding apparatus need to be developed and perfected to match the type of food sources available. Despite the digestive tract varies in the vastly diverse order of Pisces, the functional characteristics of nutrient assimilation show some degree of uniformity, which include capturing food, secreting digestive enzymes and absorbing of nutrients (Ringø et al., 2016; Govoni et al., 1986). Other functions of the gastrointestinal tract include osmoregulation (Varsamos et al., 2005), secretion of hormones not only involved in regulate digestion (Buddington and

Krogdahl, 2004), and protection of the organisms from pathogens that may reach the alimentary tract with the food and water (Yukgehnaish et al., 2020). Thus, the digestive tract represents a critical interface between the internal and external environments of the fish.

During early life stage, newly hatched larvae possess a very underdeveloped digestive system and major changes in digestion, absorption, transport and assimilation of nutrient requirements are occurring before reaching the full digestive capacity. As such, it is no surprise that nutritional requirements and feeding behaviour in fish larvae is very different from the juvenile and/or adult forms, showing the importance of studying the development of the larval digestive system (Holt, 2011; Yúfera and Darias, 2007; Rønnestad et al., 2007; Morais et al., 2007; Cahu et al., 2009; Gisbert et al., 2009, among others). This is fundamental for development of a sustainable production of fish larvae in captivity.

From a morphological point of view, the alimentary canal of the larva at hatch appears as a straight tube placed next to the yolk sac. While larvae are initially survive on the resources included in the yolk sac (endogenous feeding), the gut goes through quick developmental changes involving the differentiation of several regions and organs of the digestive system namely buccopharynx, esophagus, intestine, pancreas, and liver preparing the larva for the onset of the first feeding (Falk-Petersen, 2005). In most marine fish larvae, liver, pancreas, and gallbladder are functional already shortly after hatch (Rønnestad et al., 2007). At the same, enzymes for the luminal digestion of proteins (e.g. trypsin and chymotrypsin), lipids (e.g. lipases and phospholipases), and carbohydrates (e.g. amylases) have been detected in newly hatched larvae (Kolkovski, 2001). In addition, alkaline proteases play a major role in digestion during the first day of exogenous feeding, while acid proteases become increasingly important toward the end of the first feeding period larval period, simultaneous with the appearance of a functional stomach (Zambonino Infante and Cahu, 2007). The release and activity of those digestive enzymes and their modulation through dietary nutrients are controlled by hormonal mechanisms (Rønnestad et al., 2007). For examples, gastrointestinal hormones as cholecystokinin (*cck*) play an important role not only in the stimulation of pancreatic enzyme secretion but also in gallbladder contraction, intestinal peristalsis, and gut transit time in fish larvae (Micale et al., 2014). While in first feeding larvae the production of *cck* is only genetically encoded, in older larvae it seems to be regulated by dietary factors such as protein levels and dimensions (Cahu et al., 2004). Similar mechanisms applied for proopiomelanocortin (*pomc*), ghrelin (*ghrl*) and neuropeptide y (*npy*), where the gene expression is related to food intake and appetite regulation. Therefore, even though the development of the digestive system is mostly genetically preprogrammed, the diet can directly influence nutrient digestion and absorption of nutrients, and consequently, larval performance and growth.

Intake of food is prerequisite to growth and fish larvae are an great example of that amounts matters. In this regard, fish larvae grow extremely rapidly and, therefore, the total ingestion of nutrients must be high. In cod larvae, for example, growth rates of up

to 30% per day have been measured (Otterlei et al., 1999), while some species such as African catfish (*Clarias gariepinus*) may grow up to 100% per day (Appelbaum and Kamler, 2000). The growth rate of fish is influenced by availability of nutrients (Li and Gatlin, 2006). As such, growth relies on protein deposition that is mainly regulated by the growth hormone/insulin growth factor system (*gh/igf*) (Canosa et al., 2007). The effect of fasting and malnutrition on the *gh–igf* axis is related to the absence of specific nutrients, or indirectly to nutritionally induced changes in hormonal status (Pérez-Sánchez, 2000). Protein metabolism in fish larvae has been shown to be influenced strongly by the dietary amino acid profile, protein (or other forms of dietary nitrogen) digestibility, as well as dietary lipids (Valente et al., 2013). Growth performances of fish larvae are sub-optimal and nitrogen retention low when diets are imbalanced in amino acid (AA) profiles (Aragão et al., 2004). Amino acid imbalances may also cause higher mortalities (Felip et al., 2012), and skeletal deformities in fish larvae (Boglione et al., 2013). Several studies have also demonstrated that protein digestibility largely determines protein retention and growth (Rønnestad et al., 1999; Conceição et al., 1997). In particular, complex proteins, such as those present in fishmeal, are poorly digested, while free amino acids, peptides and hydrolysed proteins are normally better utilized by larvae (Rønnestad and Conceição, 2012). Lipid formulations are also a core ingredient for fish larval feed (Izquierdo et al., 2000; Rainuzzo et al., 1997; Sargent et al., 1999). Nevertheless, Morais et al. (2007) observed that in Senegalese sole a diet with a higher lipid content affected gut morphology, amino acid metabolism and led to a lower growth rate. In general, deficiencies in these nutrients are often documented to cause large larval mortalities (Hamre et al., 2013). Thus, the feed composition, in particular the imbalance of specific ingredients such as protein, amino acids and lipids, influences the hormonal mechanisms related to the *gh/igf* system and can therefore hamper growth.

2. European eel

2.1 Life cycle

European eel, *Anguilla anguilla*, is facultative catadromous and probably semelparous fish species. Its life cycle consist of oceanic and continental phases, where parts of the formed remains obscure (**Fig. 1**). As such, the spawning site of this species has been delimited to the Sargasso Sea by Danish marine researcher, Johannes Schmidt early in the 20th century (Schmidt, 1923). This assumption is supported by the presence of early stage larvae while neither spawning nor developing adults have been traced. Thus, knowledge on the natural reproductive development, spawning, as well as egg, embryonic and yolk sac stages is lacking, while information on early feeding larval stages is scarce (Tesch, 2003). Their feeding larval stage called “leptocephali” characterized by a narrow head and leaf-shaped body are pushed by currents, such as the Gulf Stream and North Atlantic Drift, back to the continental shelves along Europe and North Africa. This 6000 km migration is takes app. 200-300 days during which the leptocephali reach a size of 45-75 mm (Munk et al., 2010). By the end of their journey, the larvae metamorphose into the transparent juvenile stage called “glass eels”, during which they enter into rivers and lakes or remain in the coastal zone. Once they adapt to freshwater or brackish habitats, they gain pigmentation and transform into so-called “elvers” and then “yellow eels” (Tesch, 2003). The growth period in freshwater and coastal habitats varies being 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.

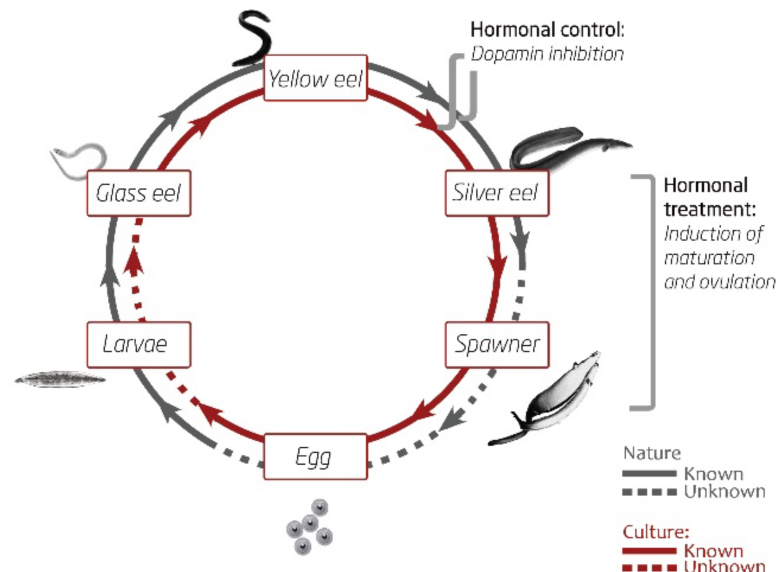

Figure 1 Life cycle of the European eel, *Anguilla anguilla* including life history stages known and unknown in nature and in culture. Graphics by Jonna Tomkiewicz and Sune Riis Sørensen, DTU Aqua

The silver eels leave the European continent in autumn mainly around new moon (Boëtius, 1980; Bruijs and Durif, 2009) and start their long migration to the Sargasso Sea, helped by sensing Earth's magnetic field (Durif et al., 2021, 2013). A set of morphological changes are involved in the migration aiming at reducing visibility to predators and promoting swimming in ocean. These changes includes altered skin pigmentation, increased eye size and visual characteristics, cease of feeding and consequent degenerate of the digestive system (Arai, 2016). The development of gonads likely occurs during the migration to the Sargasso Sea (Dufour and Fontain, 1985) and may be triggered by swimming (Righton et al., 2012). Two antagonist hormones control the initiation of gonadal maturation process: the gonadotropin-releasing hormone (GnRH), which is stimulating the pituitary to produce gonadotropins, i.e. follicle stimulation hormone (FSH) and luteinizing hormone (LH), and the dopamine, which is inhibiting it (Vidal et al., 2004). During silvering, a strong dopamine inhibition occur, blocking the gonadal maturation at a pre-pubertal stage. To go through gonadal development, this inhibition need to cease, allowing release of gonadotropins and subsequent synthesis of sex steroids, which eventually will stimulate oogenesis and spermatogenesis in the ovaries and testis, respectively.

2.2 Wild stock and aquaculture

The complexity of the life cycle together with anthropogenic stressors such as overfishing, pollution, loss of habitat degradation and climatic changes are among the causes which have contributed to the decline of the European eel stock (**Fig. 2**) (Bonhommeau et al., 2008; Dekker, 2003; Friedland et al., 2007; Gutiérrez-Estrada et al., 2020; Knights, 2003; Thillart et al., 2009). Consequently, glass eel and yellow eel recruitment have strongly dropped since the 1960s and nowadays glass eel recruitment is only the ~0.5% compared to 1960-1970s (ICES, 2020; Sonne et al., 2021). For this reasons, European eel is listed as "critically endangered" by the IUCN Red List (Pike et al., 2020).

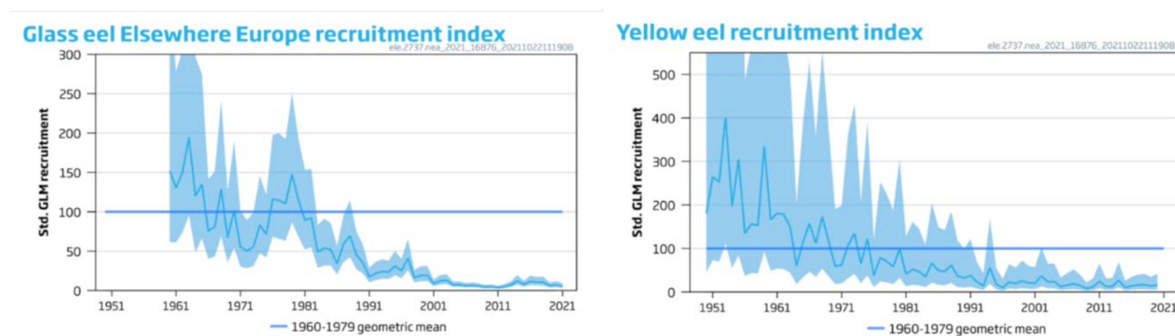

Figure 2 European eel recruitment index for glass eel and for yellow eel in Europe from 1945 to 2021 (from ICES, 2021).

Aquaculture plays a key role in sustainable market development by virtue of its potential to contribute to increased food production while helping to reduce pressure on natural

fish populations as fishery resources (OECD, 2022). This development relies on closing the life cycle for fish species in captivity, thereby replacing capture-based fish farming (Teletchea, 2015). Still, eel farming is a capture-based industry that relies on wild-caught glass eels for grow-out. Thus, the aquaculture industry work actively for establishment of hatchery technology, which would enhance markets and reduce the fishing pressure. In terms of glass eels, legal fisheries comprised 60 t annually in 2017 -2020 (ICES, 2021), however, illegal catches were estimated to 100 t for the Chinese black market (UNIPOL, 2021). One kg of glass eels comprises ~2500 eels, i.e. 160 t comprises 400 million individuals. The glass eels are legally fished primarily by France 48.7 t, Spain 6.3 t and England 3.4 t using different gears: vessel-based push nets (trawl), hand-held nets and passive gears, e.g. fyke nets (Nielsen and Prouzet, 2008). Glass eels are 6-8 cm long, glassy and fragile. Substantial mortality is caused by catch injuries, 31-42% documented in trawl fisheries, handling, caging to high-grade prices and transportation adds to this and dead upon arrival is estimated to 2-10 %. Therefore, the development of eel hatcheries supplying eel aquaculture with glass eels would enable a sustainable production with enhanced efficiency, while independent of wild-caught glass eels would support the critically endangered European eel (Pike et al., 2020) and help counteracting illegal trade. Thus, there is an urge to develop reproduction methods and larval culture technology for glass eel hatcheries.

2.3 Hatchery development and EEL-HATCH facility

The first successful induction of reproductive development was in male eels date back in the 1930s (Boucher, 1934; Fontaine, 1936) while the female ovarian development and production of eggs was developed approx. 30 years later (Boëtius and Boëtius, 1967; Fontaine, 1964). Further development of methods managed to enhance protocols lead to that European eel eggs and larvae was successfully produced for the first time almost 40 years ago with a larval longevity of 3.5 days (Bezdenezhnykh et al., 1983). These achievements motivated Japanese researchers and the first successful production of leptocephali and glass eel of Japanese eel, reviving the research interest developing hatchery technology for a future self-sustained aquaculture (Tanaka et al., 2001; 2003). For the European eel, the challenge was not taken up again until almost two decades later (Palstra et al., 2005; Pedersen, 2004), inspired by progress made for the Japanese eel.

Since then, hatchery technology development for European eel has made a leap, getting closer to completing the life cycle of eels in captivity (Mordenti et al., 2019; Tomkiewicz et al., 2019). Still, assisted reproduction protocols are applied to induce and maintain reproductive development under hatchery conditions, as sexual maturation of eel in their natural environment as well as in captivity is inhibited at the brain-pituitary level and no antagonist yet discovered (Vidal et al., 2004),. This includes for females the administration of salmon or carp pituitary extracts as source of gonadotropins to prompt oocyte development and progesterone steroid to complete the follicular maturation and ovulation. For male eels, it involves administration human gonadotropin that promotes spermatogenesis and spermiation.

The development of such standardized protocols for the European eel has enabled successful production of viable eggs (Mordenti et al., 2014; Sørensen et al., 2016b; Tomkiewicz, 2012; Tomkiewicz et al., 2019). Thus, recent studies on hormonal induction of gametogenesis, gamete-handling procedures, sperm to egg ratios and *in vitro* fertilization processes (Butts et al., 2014; Di Biase et al., 2016; Mordenti et al., 2014, 2013; Sørensen et al., 2013) provide the basis to create standardized assisted reproductive protocols, which led to high fertilization success. Successive research efforts focused on defining biotic and abiotic factors enhancing survival during embryonic incubation and larval culture (Politis et al., 2014; 2018; Sørensen et al., 2014; 2016a). This led to the first description of the early ontogeny of European eel (Sørensen et al., 2016b) from zygote until the first feeding stage. Thus, the current challenge is to establish feeding culture of European eel.

In Denmark, this challenge has been taken up during the innovation project "*Eel Hatchery Technology for a Sustainable Aquaculture*" (EEL-HATCH; www.eel-hatch.dk, 2014-2017). As part of this project a modern eel hatchery (**Fig. 3**) was established by DTU Aqua and industrial partners (Billund Aquaculture, STMI, and the North Sea Science Park). This 650 m^2 facility, EEL-HATCH, located in Hirtshals (Northern Jutland, Denmark) provides the framework for state-of-the-art eel hatchery research and technology development. In 2018, a new project called "Improve technology and scale-up production of offspring for European eel aquaculture" (ITS-EEL; www.its-eel.dk) started with the specific aim to produce enhance eel larval culture and progress towards glass eel production. This PhD project was an integral part of the ITS-EEL project.

Figure 3 EEL-HATCH facility located in Hirtshals, Denmark (Photos: *Sune Riis Sørensen*, DTU Aqua)

2.4 Captive reproduction and hormonal therapy

For both sexes, protocols of induced reproductive development require repeated treatment with gonadotropin-rich compounds to sustain gametogenesis and stimulation (steroid) of final maturation for females [12-16]. In female, the dopaminergic inhibition of gonadotropic function characterizes the continental phase of the European eel life cycle, where the production of the two main gonadotropins, i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH), is blocked (Vidal et al., 2004). The roles of these two gonadotropins is to stimulate sex steroids including estrogen (e.g. estradiol-17β, E2) via androgens (e.g. testosterone, T and 11- ketotestosterone,11-kt) and progestogens (e.g. 17α,20ß-dihydroxy-4-pregnen-3-one, DHP). Thus, FSH stimulates via androgens in the follicular cells the production of E2 involved in the hepatic synthesis of the vitellogenin, the main precursor of the egg yolk. While, LH is known to be involved in the final maturation and ovulation through stimulation of maturation-inducing steroid (MIS) (Nagahama and Yamashita, 2008).

To overcome the hormonal inhibition in captive reproduction, administration of exogenous gonadotropins of either carp or salmon pituitary extracts are applied to induce sexual maturation and vitellogenesis in female European eel in captivity. Bezdenezhnykh et al., (1983) applied biweekly injections of carp pituitary extracts (CPE) producing the first larvae of European eel. Nowadays, weekly injections of either salmon (SPE) (da Silva et al., 2016; Pedersen, 2003; Tomkiewicz, 2012) or carp pituitary extracts (CPE) (Mordenti et al., 2014; Palstra et al., 2005; Pérez et al., 2011) are used to induce vitellogenesis. When female eels are injected with either carp or salmon PE at dosage of around 20 mg kg-1 initial body weight, spawning will occur after 12-20 weeks (e.g. Kottmann et al. 2020a). However, the administration of PE alone is not sufficient to complete oocyte growth: the an extra injection of maturation inducing steroid progestogen (DHP), the most commonly used MIS, is necessary to complete final follicular maturation and ovulation, often preceded by a priming dose of PE (Nagahama and Yamashita, 2008). For male eel, weekly injections of human chorionic gonadotropin (hCG) are used to induce spermatogenesis and five injection are enough to reach spermiation (Asturiano et al., 2006; Perez et al., 2000). However, highest efficiency and sperm quality may be obtained through weekly injections, with or without a priming dose administered 24 h before stripping (Koumpiadis et al., 2021; Ohta et al., 1997).

Figure 4 Broodstock assisted reproduction methodologies (Photos: *Sune Riis Sørensen*, DTU Aqua).

3. European eel offspring culture: State-of-the-art

3.1 Egg and larval culture: incubation and larval rearing

Natural spawning and *in vitro* fertilization procedures for European eel have been successfully established for production of viable embryos (Butts et al., 2014; Di Biase et al., 2016), while suitable bio-physical conditions in the ambient environment, which are vital for survival throughout embryonic incubation and early larval rearing have been identified. Here, stage specific requirements have been experimentally determined for yolk sac larvae, identifying tolerances and optima related to for instance temperature, light, and salinity (Politis et al., 2021; 2017; 2014). These results are complemented by the development of suitable rearing systems, resulting in high survival to the first-feeding stage, enabling experimental research on the early life stages that remain undiscovered in nature (Sørensen et al., 2016a). Here, physiological research, identifying the molecular ontogeny of the early larval immune system (Miest et al., 2019), sensitivity to microbial interference (Sørensen et al., 2014) and endocrine control of larval feeding biology, has promoted the successful transition to exogenous feeding larvae (Butts et al., 2016; Politis et al., 2018b). These procedures, developed and applied at the EEL-HATCH facility, have been the backbone of the studies included in this PhD project and are summarized in the next paragraphs.

Fertilization procedures involve manual stripping of eggs and *in vitro* fertilization with pre-stripped milt stored in an immobilizing medium (Peñaranda et al., 2010). A standardized egg to sperm ratio is aimed at optimized conditions for every female in order to reduce noise in experimental tests. A pool of sperm from 3 to 5 males is commonly used during the fertilization procedures to ensure the highest fertilization rate (Sørensen et al., 2016b). Then, fertilized eggs are incubated in 60 L black conical incubators (**Fig. 5**), supplied with water at ~36 psu and ~18°C (Politis et al., 2017). Light is kept at low intensity (Politis et al., 2014), gentle aeration is applied and sinking dead eggs are purged from the bottom valve of each incubator. Approximately 48 hours after fertilization (hpf), aeration is stopped to allow larvae to hatch, which occurs at ~56 hpf.

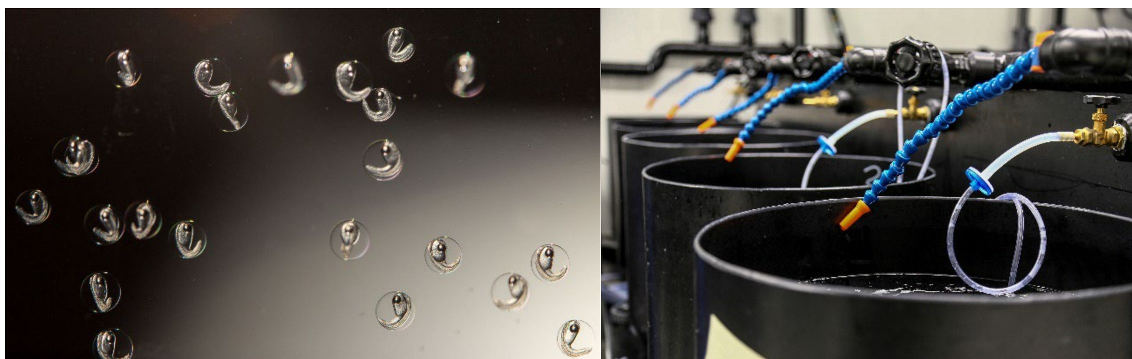

Figure 5 Embryos of European eel and incubators at the EEL-HATCH facility (Photos: *Sune Riis Sørensen*, DTU Aqua)

At hatch, European eel larvae are relatively undeveloped with distinct yolk sac and a large perivitelline space as well as an oil droplet, contributing to attaining neutral buoyancy. When eel larvae are cultured at ~20°C, eye pigmentation and lower jaw formation are visible around 8 dph (Sørensen et al., 2016). The feeding apparatus with characteristic, protruding teeth is formed around 10 dph (Politis et al., 2018a), while the yolk sac is completely exhausted around 13 dph (Bouillart et al., 2015; Sørensen et al., 2016). Larval development throughout the yolk sac stage is illustrated in **Figure 6**.

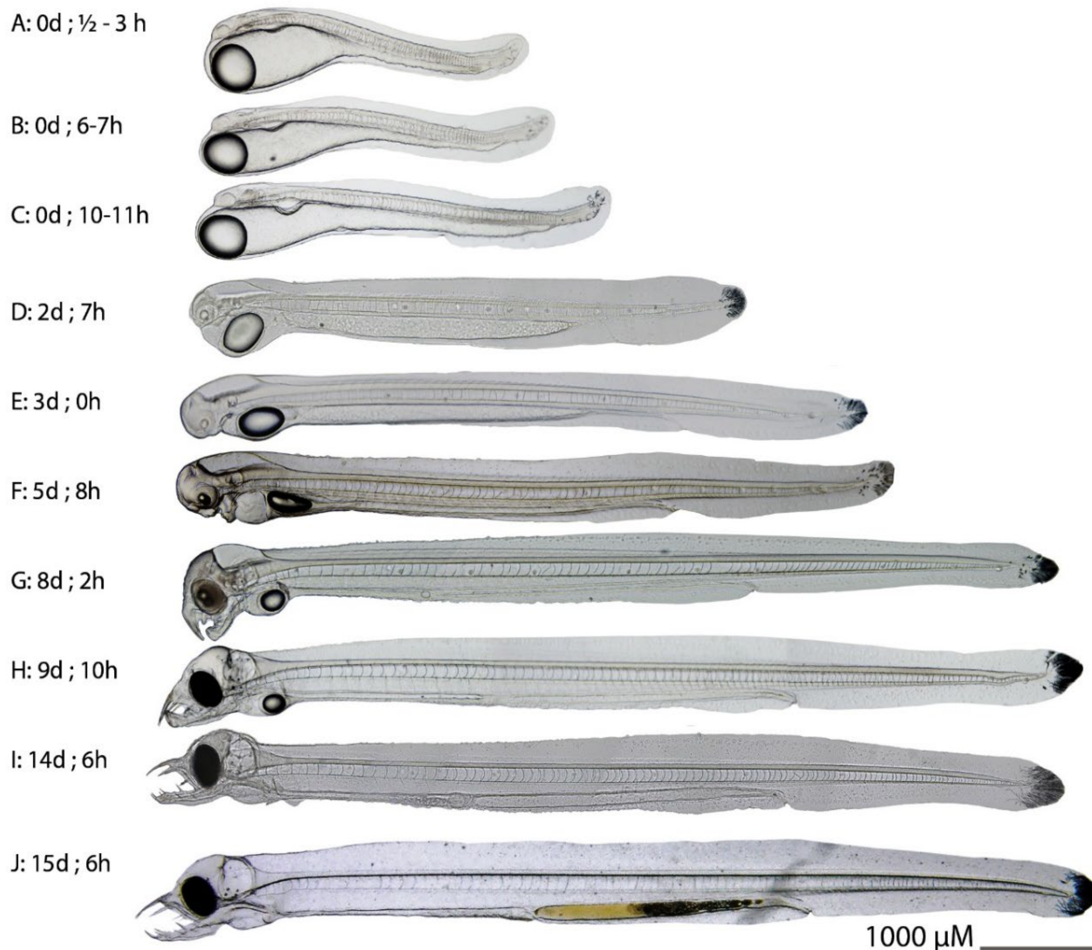

Figure 6 Developmental stages from hatch to first feeding stage of European eel (*Anguilla anguilla*) larvae reared at 20°C. From Tomkiewicz et al., 2019.

Several experimental studies have investigated suited rearing conditions, including biotic (microbial activity) and abiotic factors (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 and hatch success as well as larval survival. Thus, eel embryos and newly hatched larvae need to be reared in clean water with a very low bacterial load. Additionally, eel larvae have been shown to be sensitive to light, where larval culture with a light intensity higher than 1000

lux showed low survival (Politis, et al., 2014). Furthermore, European eel larvae cultured at 18-20°C showed higher growth efficiency and lower abundance of deformities than larvae at colder or warmer temperatures (Politis et al., 2017). In addition, the reduction of salinity towards iso-osmotic levels (18 psu) has proven to increase survival and facilitate growth (Politis et al. 2018c). However, reducing the salinity at the wrong developmental time may provoke severe deformities, in particular heart edema. Therefore, further investigations were necessary to understand how to reduce the salinity and at which developmental stage (Politis et al., 2021; Syropoulou et al., 2022). Based on the results of these studies, the authors concluded that eel larvae can tolerate a drastic change in salinity (from 36 to 18 psu, applied within few hours) and that the best time to reduce salinity is at the late yolk sack stage around 9-10 days post hatch.

3.2 Eel larval feeding

Developing a suitable first-feeding diet for European eel is currently the main focus in eel larviculture research. Despite increasing insights relying on advanced analyses of stomach content, the natural diet of eel larvae is not fully uncovered (Ayala et al., 2018; Miller et al., 2019; Riemann et al., 2010; Tomoda, 2018). At the same time, research towards developing suitable first feeding diets for hatchery produced eel larvae has been very active. Here, larvae of Japanese eel are currently fed a slurry diet based on shark egg yolk (Tanaka et al., 2003), hen egg yolk, exoskeleton-free Antarctic krill (Okamura et al., 2013), or a protein hydrolysate based diet (Masuda et al., 2013).

For European eel, tests regarding the identification of suitable feeds for larval culture, targeting stage specific nutritional requirements, are still in a pioneering phase. The first insights on potential feed items was revealed by investigating the morphology of the feeding apparatus. Here, the biting force and size of ingestible particles were estimated by histological 3D-reconstruction of the somatic structure of the feeding apparatus (Bouilliart et al., 2015). Still though, the distinct attachment sites of the first pair of teeth, in both the upper and lower jaw, indicate that these teeth are involved in biting only small and soft food particles. As a combined result, a rather weak bite force (± 50 µN) and a relatively small maximum gape angle of the lower jaw (± 25°) suggest a preference for very soft and/or small food organisms and/or particles (Bouilliart et al., 2015).

In vivo, first results by Butts et al. (2016) showed that up to 50% of cultured larvae in the experiment ingested a diet composed of enriched rotifers (*Brachionus plicatilis*), concentrated and emulsified into a paste, with or without chemo-attractant stimulants (**Fig. 7**). Moreover, it was documented that first-feeding eel larvae are able to execute a complex goal-oriented motor response, where highly distinctive modes of swimming were observed from short-term bouts, slow steady state cruising, to quick lunges for either prey attacks or spontaneous escape behaviours. Swimming activity increased over time, co-varied with the frequency of attacks and increased in the presence of live rotifers or chemo-attractants, probably by increasing the awareness of food availability. Furthermore, improved ingestion was detected at higher light intensities, suggesting that these larvae are visual feeders, which complies with their large eye globules. However,

the observed chemo-attraction and successful food intake, with and without light, indicate that this species is able, besides using visual cues, to utilize other stimuli (olfaction, taste) to detect prey (Butts et al., 2016).

Thereafter, utilizing the diet developed in Butts et al., 2016, a study investigating the endocrine regulation of feeding, during the transition from endogenous to exogenous feeding, identified the first feeding window and described a genetically pre-programmed feeding mechanism with molecularly early maturing digestive functions (Politis et al., 2018a). Here, despite the successful initiation of first feeding at 15 dph (cultured at 18°C) and an improved body area compared to starving conspecifics, the larvae did not survive past 24 dph. However, eel larvae expressed genes related to digestion and appetite already at 4 dph, indicating an adaptive potential towards a prompt maturation of the gastrointestinal function. Although the diet did not enable larval survival and growth, the study significantly enhanced insights regarding eel larval feeding biology and provided a benchmark diet for further feeding trials (Tomkiewicz et al., 2019).

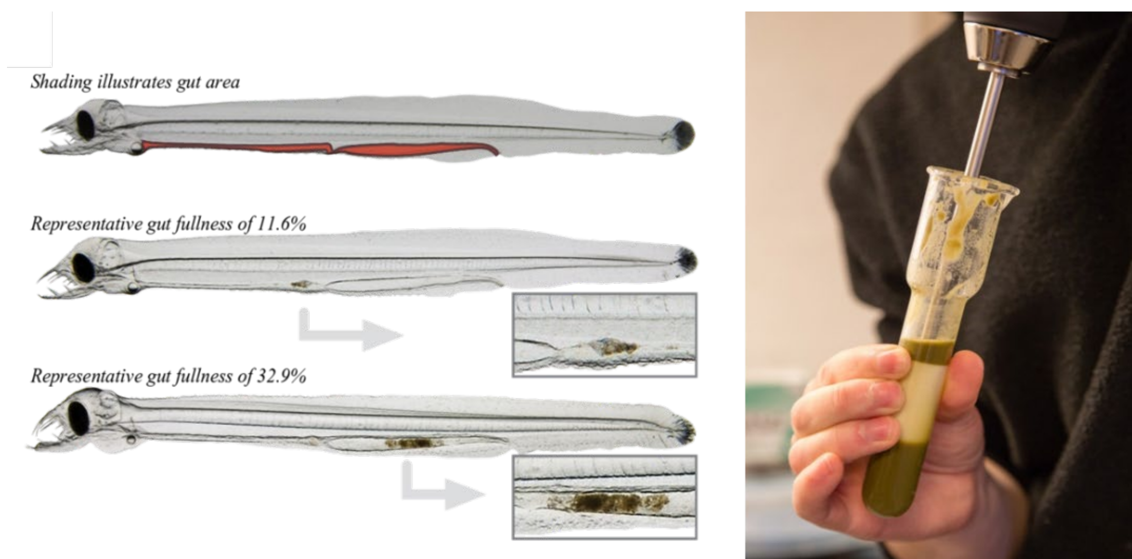

Figure 7 Left: European eel larvae feeding on rotifer paste. Orange shading represents gut area, with gut-fullness of 11.62% and 32.9%. From Butts et al., 2016. Right: emulsifying rotifer paste. (Graphic design and photo by *Sune Riis Sørensen*, DTU Aqua)

4. Methodologies

An array of different methodologies were utilized in this PhD project combining experimental records and morphological measurements through image analysis with biochemical analyses and molecular tools, thus, applying a multidisciplinary approach to obtain novel insights and integrate findings.

The first study combined biochemical composition analyses with morphometric measurements to assess quality of eggs and yolk sac larvae of European eel. As the nutritional quality of larvae plays a key role in the survival until first-feeding, analyses of biochemical composition of eggs and larvae were the first step. Here, the impact of assisted reproductive protocols on offspring quality and developmental capacity was assessed. The initial amount of nutrients in the egg and the subsequent depletion of the yolk resources during embryonic and larval development are important to survival and developmental success. This applies to both the nutrient utilization for incorporation into larval tissues and as energy, which together provide information about the requirements and relative importance of nutrients for the developing larvae.

Also the diets used in Study 3 and Study 4 were biochemically analyzed in terms of macronutrients such as protein, lipid, ash and dry matter. This information about the biochemical composition was part of tailoring the diets and evaluating the eel larval response on the different diets.

Furthermore, molecular tools were integrated into study 2-4 to enhance insights into the complex processes of early ontogenesis as well as the maturation of main functions during the organism’s development. Here, the recently sequenced genome of European eel, first, the annotation by Henkel et al. (2012) and subsequently, the version at chromosome level by Rhie et al. (2021) has been an advantage. Here, the use of Fluidigm technology enabled the analysis of expression of multiple genes involving multiple samples at the same time.

This PhD project used gene expression technology to better understand physiological responses of the early life history stages of European eel in relation to nutritional drivers. Knowledge on the expression of genes related among others to appetite, food intake, digestion and growth is valuable, as it can give important information about the nutritional condition of an organism. Here, the expression pattern of genes related for instance to digestive enzymes, naturally follows the development of the organs responsible for their production. Accordingly, the development of the digestive system and expression of related genes are genetically programmed and therefore stage specific, which makes it possible to use the expression of specific genes as biomarkers for larval developmental success. This is particularly interesting in larval culture development, where suboptimal larval diets could potentially provoke a reduction of performance and high mortalities.

In addition to the molecular development of growth, appetite and digestive capacity in relation to nutritional factors, immune and stress/repair processes have been targeted to

increase knowledge regarding larval physiological response to the additions of nutrients to the culture. Together, the gene expression of these genes enable the description of characteristics of successfully developing prefeeding and feeding European eel larvae.

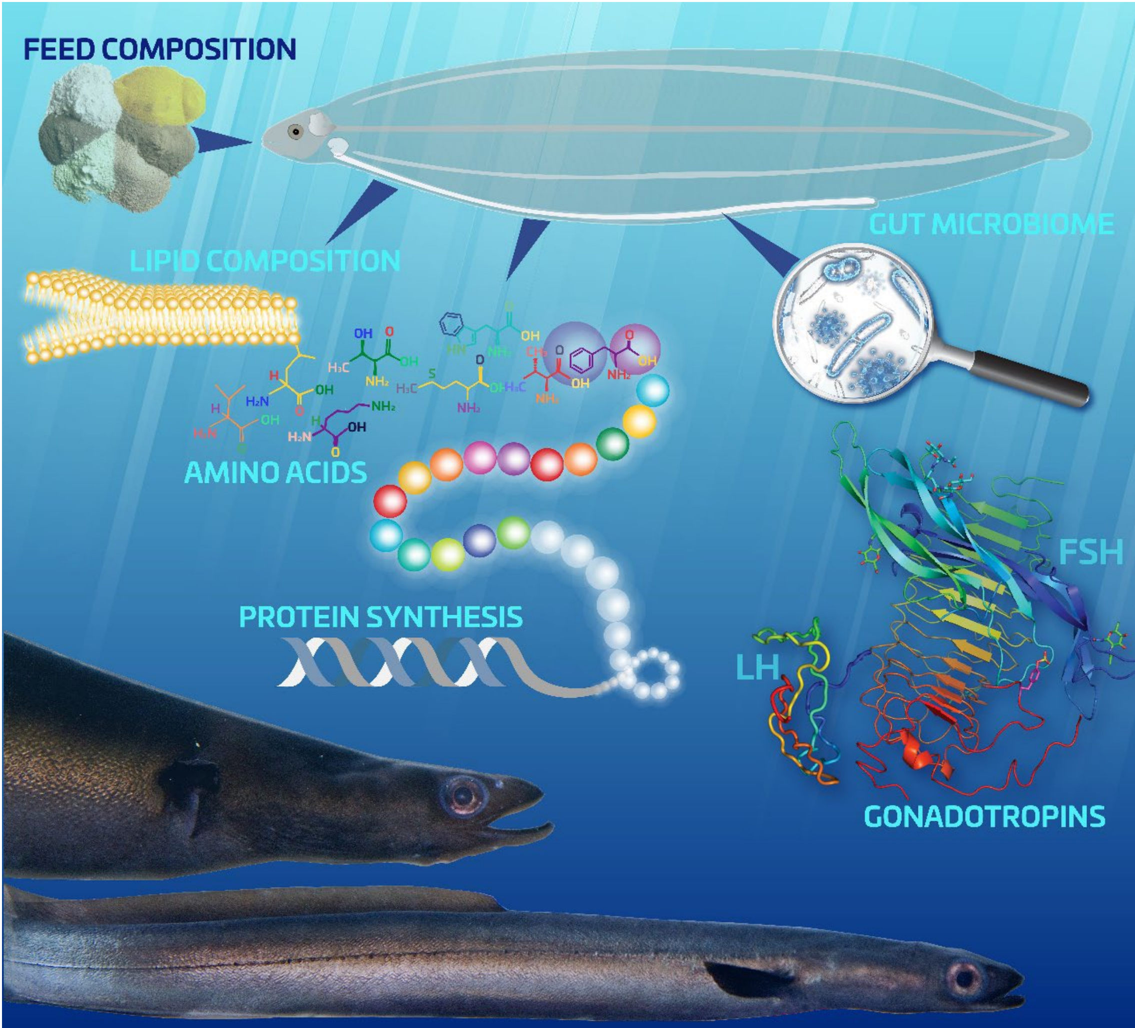

Figure 8 Schematic representation of the targets addressed by the methodologies used in this PhD project (Graphics design by Sune Riis Sørensen, DTU Aqua).

5. Gaps in knowledge

In recent years, the main bottleneck in the development of European eel aquaculture has become the establishment of a first feeding larval culture and on growing of leptocephalus larvae. Here, larval survival after the exhaustion of the yolk sac require nutrient intake but no suited feeds have been developed yet, so longevity is still restricted. Several factors needs attention, which include larval quality in terms of yolk sac reserves, rearing system design and functionality, larval nutritional requirements as well as host-environment-microbial dynamics in feeding larval culture. The present PhD project focused on two of these factors, i.e. the larval quality and feeding regime.

In order to establish larval culture of European eel, the highest quality of embryos and yolk-sac larvae need to be ensured. Rearing conditions for early life stages have been partly tested and established, but **high variation in survival** between batches is still affecting production. Considering that rearing conditions at the EEL-HATCH facility are kept constant, factors influencing offspring production and quality may be related to the broodstock. Among those factors are potential **effects of assisted reproduction on egg and yolk sac larval quality**. Currently, European eel reproduction protocols are based on hormonal treatments, including application of exogenous gonadotropins, which are required to facilitate the production of viable gametes. Such hormonal applications, although serving the purpose, may have unintended species-specific impacts on gamete and offspring quality (Mylonas et al., 2010). Hormonal treatment protocols for the induction of vitellogenesis in female European eel include weekly injection of either carp or salmon pituitary extracts (CPE or SPE). Here, the composition of applied pituitary extracts, including contents of active gonadotropins (FSH and LH), and their potential effect on egg and offspring quality is not fully explored. In a previous study by Kottmann et al. (2020b), differences in reproductive success and embryonic quality were observed in response to female hormonal treatment with SPE and CPE. However, the hormonal treatment may also affect the biochemical quality of eggs and yolk-sac larvae. In this regard, the first study (**Study 1**) of this PhD project compared the influence of CPE and SPE treatments on biochemical composition and nutrient utilization of offspring. The amount of total lipids, protein, amino acids and fatty acids were assessed in fertilized eggs and larvae throughout the yolk-sac stage, while body and oil droplet area were measured to estimate growth rate, oil droplet utilization and oil-droplet utilization efficiency. These stages were chosen as embryos and early yolk-sac larvae survive solely on resources comprised in the yolk (and oil droplet), embedded in the developing oocytes during vitellogenesis and therefore depending on maternal condition.

The second aspect taken into consideration in this PhD project is the eel larval feeding requirements. Here, knowledge is scare regarding suited diet composition, attractiveness and consistency as well as feeding regimes. The first questions relate to factors affecting the successful transition from endogenous to exogenous feeding, where undeveloped gut functionality may hamper successful feeding. The gastro-intestinal tract in fish is important for digestion and assimilation of food, and in this regard, the microbial

community harboring the gut, acting as an integral component of the host, plays key roles in the modulation of the immune system, the proliferation of the intestinal epithelium and the regulation of the dietary energy intake. In fish larval culture, gut microbiota can be driven by the use of gut priming agents such as *probiotics* (defined as live microorganisms promoting health), *prebiotics* (compounds stimulating growth and activity of the gut microbiome), or *synbiotics* (formed by the synergistic effect of pro- and prebiotics). Therefore, **Study 2** tested the effect of commercially available gut-priming agents (probiotics, prebiotic and synbiotics) on eel larval development and survival. Furthermore, the expression of genes related to appetite, food intake, digestion, and growth to explore effects on the maturation of gut functionalities and the transition towards exogenous feeding. Furthermore, gene expression analyses included immune and stress responses during the endogenous feeding stage with the intent to investigate further the larval physiological responses to the change in the environment.

Similarly, **Study 3** of this PhD project targeted physiology-driven insights into gut maturation through introduction of feeds prior to reaching the exogenous feeding stage. As the natural food and location of the earliest eel larval stages remains unknown, experimental research is necessary to obtain insights into the window of opportunity within which fish larvae should start actively to search for food and identify, ingest and digest suited feed items. From other fish larvae, it is known that if feeding does not occur within this window, larvae enter the so called point of no return and suffer irreversible starvation. To learn about this critical larval phase, Study 3 investigated the effect of introducing feed as early as 4 dph, at a developmental stage where recent research has shown that digestion related genes are already being transcribed (Politis et al. 2018). The study was explored the influence of food composition on the molecular ontogeny of eel larvae by formulating several diets, as no diet has yet proven effective for European eel larvae. Altogether, three pioneering trials were performed, where three different formulated diets and feeding regimes were applied to test the hypothesis that an early introduction of feed can improve gut maturation and enhance digestion capacity. In order to enhance the physiological understanding, the expression patterns of genes related to appetite, food intake, digestion, and growth as well as immune and stress response were analysed prior to and throughout the first-feeding stage, complementing growth performance and survival measurements.

The available knowledge regarding natural European eel larval feeding preferences and nutritional requirements is negligible, and most available information has been obtained through experimental research covering studies of rotiferbased inert diets, diet consistency, feeding behavior, and light during feeding (Butts et al., 2016; Politis et al., 2018b). Here, larvae successfully ingested a paste made of rotifers, *Brachionus plicatilis* (Butts et al., 2016), but despite successful initiation of first feeding and larger body area than starving conspecifics, the larvae did not survive past 24 dph (Politis et al., 2018b). On the other hand, progress regarding feeding regimes for the closely related Japanese eel (*A. japonica*), applying diets based on spiny dogfish egg yolk (Tanaka et al. 2001; Tanaka et al., 2003), has led to a closed cycle production of larvae and glass eels (Tanaka, 2015). In this context, **Study 4**, combining insights gained from previous

European and Japanese eel research, innovative diets, formulated in collaboration with Sparos Ltd, inspired by the Japanese recipe were formulated and tested as potential first feeding diets for hatchery produced European eel larvae. The experiments recorded larval survival and performance via morphometric measurements, while the expression patterns of genes related to appetite, food intake, digestion, and growth as well as energy metabolism were explored to explore and evaluate effects on underlying physiological mechanisms. Obtaining information about the earliest feeding stages and their physiology can fill gaps in knowledge of outmost importance for the establishment og feeding larvae culture of the European eel, by providing evidence of nutritional requirements and biomarkers for healthy development

Figure 9 - Sampling larvae of European eel *Anguilla anguilla* from experimental units (Photography Sune Riis Sørensen, DTU Aqua)

6. Findings of my PhD

Study 1: Egg and larval quality

Type of hormonal treatment administered to induce vitellogenesis in European eel influences biochemical composition of eggs and yolk-sac larvae

In this study, the biochemical composition and morphological records were compared for eggs and yolk sac larvae produced through assisted reproduction of broodstock using two different protocols.

Assisted reproduction protocols for female European eel involved weekly administration of pituitary extracts from carp (CPE) or salmon (SPE) to induce and sustain vitellogenesis. During vitellogenesis, nutrients are deposited in the oocytes, composing the yolk that later becomes vital for the survival and development of eggs and larvae. Here, if an egg does not contain the appropriate types or amounts of nutrients, embryonic development might be hampered and, consequently, larvae might not reach the first feeding stage. This study process investigated if the nutrient deposition was differentially influenced by the two hormonal treatment.

Dry weight, proximal composition (total lipid, total protein), free amino acids (FAA) and fatty acids of eggs, embryos and larvae were analyzed and the pattern of nutrient utilization during the yolk-sac stage followed to gain insight into the nutritional requirements of eel larvae during the transition during early development.

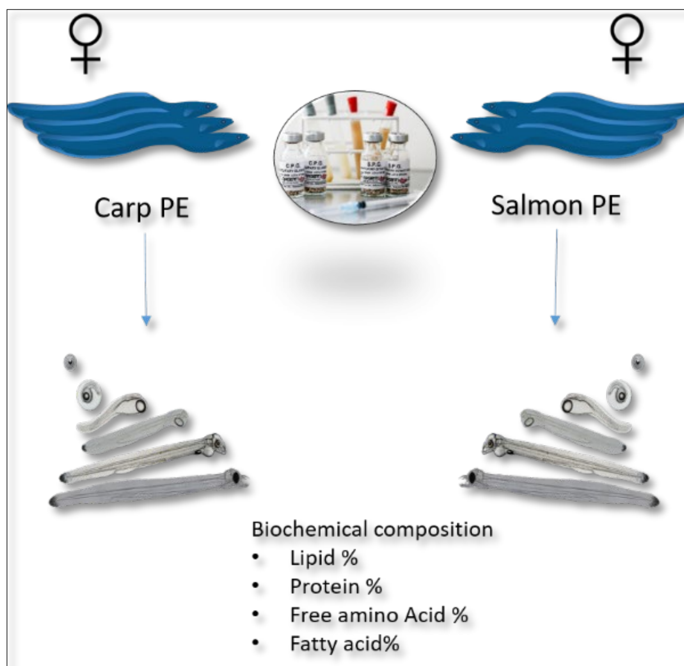

Figure 10 - Simplified schematic of the experimental set-up regarding the effect of hormonal treatment on biochemical composition of eggs and yolk sac larvae of European eel.

Also, body and oil droplet area were measured to estimate quality in terms of growth rate, oil droplet utilization and oil-droplet utilization efficiency in eggs and larvae throughout the yolk-sac stage.

The results showed that CPE females spawned eggs with higher lipid and free amino acid contents. On the other hand, SPE females produced a higher quantity of eggs with higher fertilization rate as well as larger larvae with more energy reserves (estimated as oil-droplet area). Thus, the assisted reproductive protocols significantly influenced offspring quality. Nevertheless, towards the end of the yolk-sac stage, the proximal composition and biometry of surviving larvae from both treatments were similar. This study, once more, highlights the importance of broodstock quality and management practices for the production of high quality gametes, which is the first step to create a reliable larviculture. Noteworthy is the higher quantity of viable offspring from SPE treated females.

Moreover, the results of this study revealed general patterns of nutrient utilization, where essential fatty acids (EFA) and free amino acids were retained, while lipid and monounsaturated fatty acids (MUFA) decreased throughout the yolk sac larval stage (until 8 dph), probably being used as main source of energy. The fact that these nutrients were limited beyond 8 dph, suggests that eel larvae could benefit from supplementation of exogenous food availability around this age.

Highlights:

- Maternal assisted reproduction protocols influenced egg quantity, fertilization capacity and biochemical composition
- Treatment differentially affected offspring lipid, protein, FAA content and biometry
- While prominent for eggs and hatched larvae, biochemical differences decreased during yolk sac stage
- Lipid and MUFA significantly decreased during early ontogeny in both therapies
- Protein, FAA and EFA were selectively retained i.e. stable or increased levels

Gut priming: Study 2

First assessment of prebiotics, probiotics, and synbiotics affecting survival, growth, and gene expression of European eel larvae

In this study, the effects of gut priming agents on the survival, growth and gene expression of eel larvae were tested.

Considering that the larval gastro intestinal system is under development, priming the larvae by microbial steering with probiotics and prebiotics has been suggested to improve the larval digestive capacity as well as the immune response. Here, the immune system of fish larvae is fragile especially during the early development, rendering larvae vulnerable to pathogens in a microbe-rich environment. Yet, the microbial communities inhabiting the gastrointestinal tract play a key role in the functioning of the organism, actively participating in the establishment of feeding and digestion functionality. Thus, gut microbiome drives maturation of the gastro intestinal tract and affects the transition from endogenous to exogenous feeding.

Accordingly, commercially available prebiotics [AgriMOS, Mannan-oligosaccharides, and β-(1,3 and 1,6)-poly-D-glucose], probiotics (Bactocell, Pediococcus acidilactici), and synbiotics (AgriMOS + Bactocell) were administered to European eel larvae during the endogenous (pre-)feeding stage, following a pioneering experimental introduction of those gut-priming agents directly into the rearing water.

The results showed an early expression of genes relating to digestion, food intake and appetite, as evidenced by their basal transcript levels already on 4 dph as well as phenotypic plasticity of the expression of appetite-regulating gene ghrelin (*ghrl*) towards gut-priming agents, indicating a prospective adaptive capacity towards earlier maturation of the larval gut to enhance digestive functionality. However, despite the knowledge gained through this study, the gut-priming regimes applied did not seem suited for eel larval culture, suggesting that different microbial sub-strains or phylogenies need to be identified and carefully considered before application as probiotics.

Moreover, increased mortality in connection with synbiotics and impaired growth in connection with probiotics and synbiotics, potentially related to the high load of organic matter, probably affected water quality. At the same time, the lack of molecular responses in immune and stress/repair related genes, indicated a still immature immuno-readiness, probably caused by the lag phase between the maternally inherited protection and the gradual build-up of the larva’s own immune system. As such, water management strategies and rearing options need to be adapted for future pre-feeding and feeding regimes, to target optimized culture conditions and ensure the production of healthy offspring.

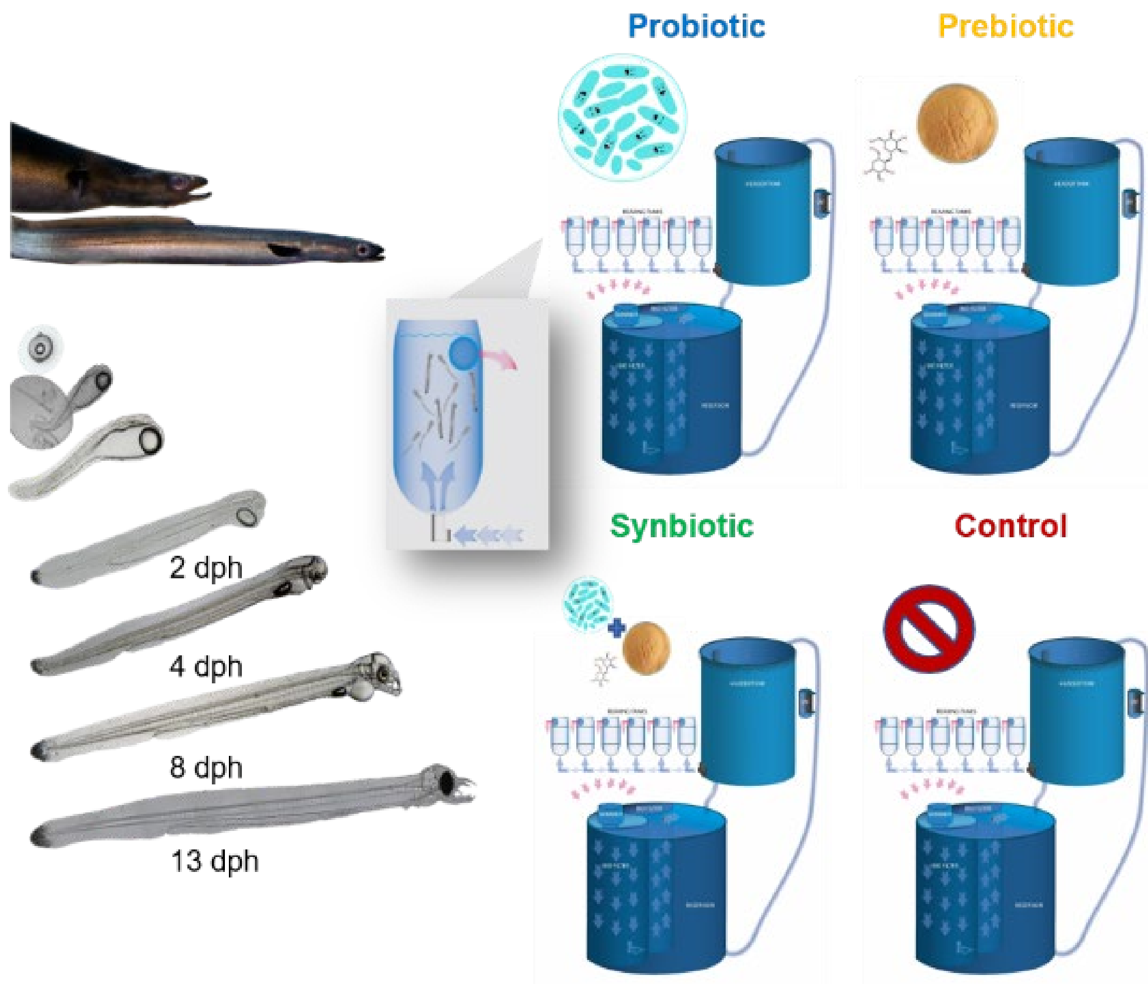

Figure 11 - Schematic representation of the experimental design of Study 2, where European eel larvae were cultivated in the presence of probiotics, prebiotics, synbiotics or with no gut-priming agents (control). (Graphic design by Sune RiiS Sørensen and Sebastian N. Politis, DTU Aqua).

Gut priming: Study 3

Effect of early introduction of feeds on the transition from endogenous to exogenous feeding in European eel larvae

In this study, pioneering diets were tested as prefeeding and as food introduction applying three different feeding regimes to explore and facilitate initiation of European eel larval first feeding.

In larviculture, mortality and poor growth often relates to dietary causes. The transitional phase between endogenous and exogenous feeding is sensitive period during which, fish larvae undergo major morphological and physiological changes. A delay in first feed ingestion can cause morphological deformities, abnormal behavior, and inability to swim or eat, thereby leading to mortality. Here, lack of food particles can be part of delaying the maturation of the gastro-intestinal tract. Therefore, identifying appropriate feeding regimes during early larval development is pivotal to produce robust animals at later developmental stages.

For European eel, establishment of first feeding culture in captivity remains a challenge. Based on the knowledge gained from previous research on cultured European eel, the first feeding window was molecularly identified between 12-15 dph (when reared at 18°C). However, expression of genes related to genetically pre-programmed feeding mechanisms were already detectable at 4 dph. Thus, we hypothesized that eel larvae might benefit from exogenous food being introduced earlier than the beginning of the first feeding window. For this, three consecutive experimental feeding trials were conducted to explore suitability of diets (Diet A-D) and feeding regimes (1-3) for first feeding eel larvae (see **Fig.12**).

The results indicated that prefeeding challenged larval survival during the endogenous feeding stage. Interestingly, results showed that the expression level of npy and cck (appetite regulation) was higher, while expression level of pomca (food intake) was lower in non-prefed larvae in Feeding regime 1 and 3, probably indicating increased likelihood of fasting and higher starvation risk. On the other hand, Feeding regime 2, showing the highest expression of growth related genes and the highest recorded survival values. Even though none of the feeding regimes seemed to provide a balanced diet to significant improve larval growth, Feeding regime 2 indicated suitable traits that need to be further developed for future European eel larviculture.

Overall, this study showed the importance of early food introduction and the sensitivity of eel larvae to different feed compositions. Finally, the results of this study increased knowledge regarding the underlying molecular mechanisms and physiological processes in relation to feeding regimes, providing core information that is important to establish a first-feeding culture not only of eel, but also of any new aquaculture species.

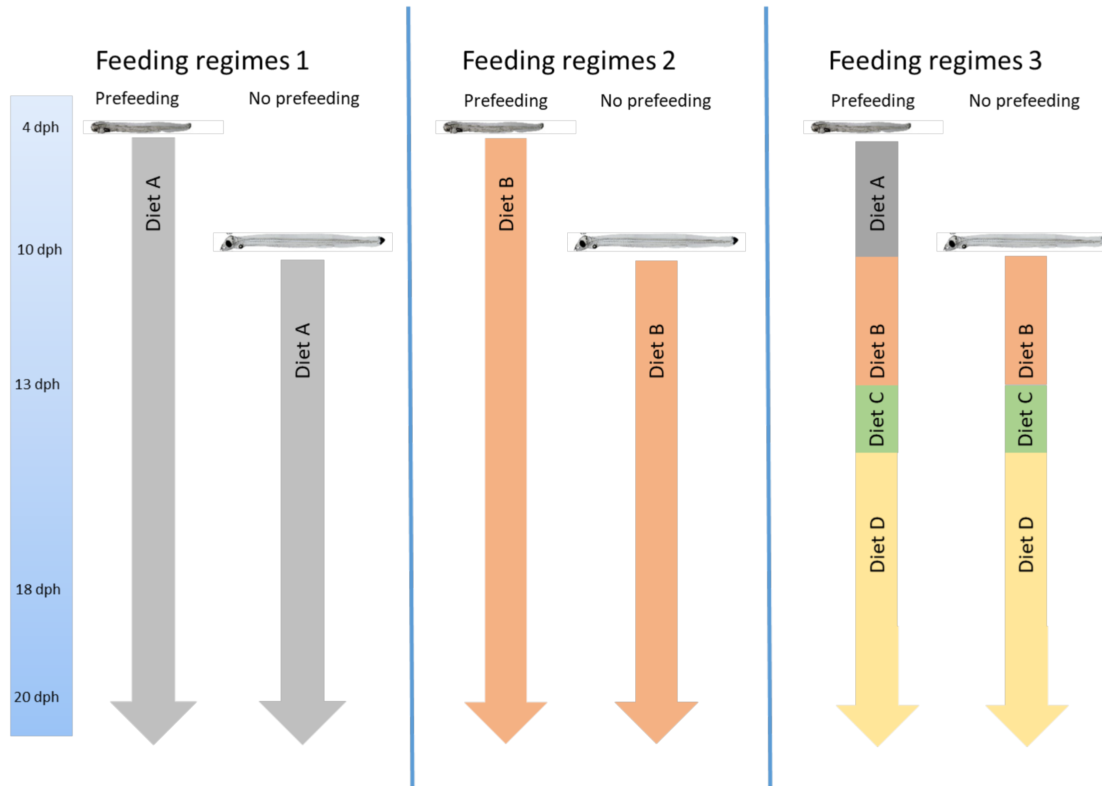

Figure 12 Schematic representation of the experimental design of Study 3, assessing the effect of early introduction of feed (prefeeding) on ingestion and digestion capacity of European eel larvae, where different formulated diets and feeding regimes were tested in three pioneering feeding trials.

Highlights

- Survival rate in Feeding regime 2 was the highest on record for European eel culture (20 dph)
- Prefeeding challenged eel larvae, resulting in reduced survival rate during the endogenous feeding stage
- In prefed larvae *hsp90* was up-regulated during endogenous feeding, while down-regulated during exogenous feeding
- Prefeeding upregulated food intake related *pomca* as well as digestion related *try*, *tgl*, and *amyl2a*.
- Expression levels of *gh* in prefed larvae exceeded those of the control during the exogenous feeding stage

First-feeding: Study 4

First-feeding regimes for European eel larval culture: Insights on morphological, nutritional, and molecular level

In this study, innovative prototype diets were formulated and tested in pioneering feeding experiments to investigate the effects on survival, growth and gene expression of first-feeding eel larvae.

Establishing the first-feeding culture of eel larvae is the key to the production of glass eels. Previous feeding attempts, using rotifer paste, did not promote growth and survival of cultured eel larvae (Butts et al., 2016; Politis et al., 2018), while an early introduction of nutritional additives may pose a risk for eel larval health (Politis et al., in prep - Study 2).

The current study, focused on nutritional aspects of eel larvae throughout and beyond the first feeding window as well as the molecular ontogeny of processes relating to appetite, food intake, digestion and growth as well as energy metabolism. For this purpose, three experimental diets were formulated, inspired by the recipe used for the closely related Japanese eel (*A. japonica*), which included among other spiny dogfish egg yolk and krill extract (Tanaka et al., 2001; 2003). Here, Diet 1 imitated the diet used to feed Japanese eel larvae, while the other Diets represented modifications of Diet 1, aiming at partially replacing dogfish egg yolk by either easening nutrient availability by the addition of fish hydrolysate (Diet 2) or increasing the molecular size of dietary protein by the addition of whey (Diet 3). Consequently, the difference between Diet 2 (~3 kDa) and Diet 3 (~10 kDa) is the molecular weight of the dietary protein.

Results showed that eel larvae successfully ingested all three diets, with feeding incidence and gut fullness increasing between 15 and 22 dph with highest gut fullness registered for Diet 3. Two periods of high mortality were identified. Firstly, at 10-12 dph with mortality probably driven by the first introduction of feed and the associated challenges related to transition to exogenous feeding and immune responses. Secondly, at 20-24 dph with mortality probably indicating that eel larvae reached the "point of no return" if feeding is not established. Here, the continuous upregulation of *ghrl* (peaking at 22 dph), the so-called "hunger hormone", indicated that most eel larvae were fasting. Interestingly though, larvae fed Diet 3, downregulated the expression of *ghrl* again beyond 22 dph, probably demonstrating that those larvae successfully overcame the challenges during this period, resulting in improved survival and growth (length, body area and dry weight) beyond the "point of no return". Moreover, the expression patterns of genes related to digestion (*try*, *tgl*, *amyl2a*), food intake (*pomca*), and growth (*gh*) continued to increase towards 28 dph for larvae fed Diet 3, underlining that this diet possesses traits stimulating the digestive processes suited for functional feeding, digestion and growth.

Furthermore, an early benefit of Diet 1 was observed, but the inclusion of more complex dietary proteins (Diet 3), but not hydrolysed peptides (Diet 2) seemed to be beneficial in order to promote larval ontogenetic performances after the successful transition to exogenous feeding. This indicates that the digestive and assimilation capacity of European eel larvae seems to vary throughout ontogeny and consequently, the dietary regime might need to be adapted according to stage specific nutritional requirements and preferences.

Overall, this study generated, for the first time, knowledge regarding the ontogeny and digestive physiology of eel larvae beyond the point of no return. Markedly, this study has has enabled the survival throughout and for the first time, beyond the first feeding stage, providing important information in relation to the nutritional requirements of European eel larvae. Therefore, these results provide a useful basis for future research efforts towards establishment of suited diets and feeding regimes for European eel larval culture.

Highlights

- Two periods of high mortality were identified at 10-12 dph after first introduction of feed and at 20-24 dph marking the point of no return.
- During first-feeding (15 dph), Diet 1 initially improved survival and resulted in bigger larvae with upregulated digestion, food intake and growth related genes.
- Beyond the first-feeding window (22 dph), Diet 3 resulted in higher survival, growth (dry weight, length and body area) and greater gut-fullness.
- Larvae fed Diet 3 showed upregulated expression of genes for digestion (*try*, *tgl*, *amyl2a*), food intake (*pomca*), and growth (*gh*) at 22 dph, increasing towards 28 dph.

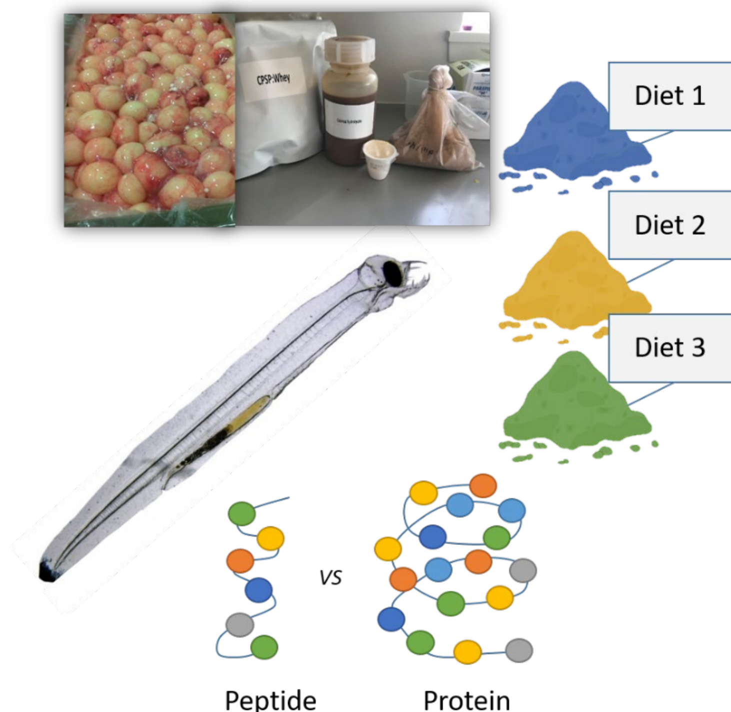

Figure 13 - Schematic representation of the experimental design of Study 4, assessing the effect of three experimental diets on ingestion and digestion capacity of European eel larvae.

7. Conclusions and future perspective

This PhD project has substantiated knowledge about the ontogeny and developmental physiology of the larval stage of European eel. The studies, at the forefront of basic and applied research, extended scientific efforts of the past ten years to establish the knowledgebase needed for embryonic and larval culture. Here, new insight regarding the biochemical composition of eggs and larvae highlighted the importance of the quality and quantity of yolk in relation to the ability of the larvae to successfully reach the first feeding stage. Moreover, the physiological understanding of the processes behind the development of the digestive capacity in relation to the transition between endogenous and exogenous feeding as well as different diets and feeding regimes has brought eel larval culture into a new phase. While more research is needed to optimize diets, the results of the present studies emphasized the complex interactions between feed addition, water quality and growth of eel larvae. Accordingly, future research in European eel larval culture must consider enhancement of larval quality and advancement of rearing techniques (water quality management) as well as improvement of dietary composition and feeding regimes.

Study 1 stressed the importance of broodstock management on the quality and quantity of offspring. Main findings include that maternal treatment influenced the egg and yolk sac larval quantity and biochemical composition. While the biochemical composition of the larvae by the end of the yolk sac stage did not differ between hormonal treatments, the production of fertilized eggs was significantly higher for SPE treated females. Yet, the results pointed to influences of the exogenous hormone administration on processes involved in the vitellogenesis and therefore, on the incorporation of yolk components. As the amount of yolk is important, future studies integrating knowledge about offspring composition and survival data are recommended to elucidate how egg quality and survival rate can be improved by adjustment of maternal hormonal treatments. Still, the physiological pathways influencing the vitellogenesis and the incorporation of the yolk components need further investigations, which might eventually lead to enhanced reproduction methods.

While high offspring quality is essential to reach the first feeding stage, it is equally important to time the introduction of dietary nutrients or additives in order to prime the digestive system. **Study 2** tested, in this context, the possibility of improving the maturation of the gut by adding non-specific probiotics or/and prebiotics to the rearing system. Results revealed an increased mortality, when eel larvae were offered synbiotics (probiotics+prebiotics), while both probiotics and synbiotics impaired somatic growth. Nevertheless, gene expression analyses indicated a prospective adaptive capacity towards earlier maturation of the larval gut, indicating enhanced digestive capacity. The gut-priming agents and regimes applied in this experiment did not appear suited for eel larvae in culture, however, also water management strategies, when adding nutrients to the larval culture, need attention. Once these issues are resolved, the application of gut priming agents might be revisited.

Study 3 used an alternative strategy, where introduction of food instead of gut priming agents was used to stimulate the gut function of the larvae. The results indicated that while pre-feeding challenged larval survival, the early introduction of nutrients might have benefited the functionality of the gastro-intestinal tract and possibly equipped the larvae with an improved digestive capacity. Although, none of the feeding regimes tested seemed to include a sufficiently balanced diet resulting in larval growth, one of the feeding regimes applied in this study resulted in the highest survival rates registered at that point in time. In fact, the larval survival rate in the treatments (prefeeding vs. control) of this feeding regime was similar at the end of the experiment (20 dph). In addition, the molecular results are showing an upregulation of genes related to digestion, appetite and food intake in particular for the best suited diet and feeding regime. The results of this study underline the potential for the use of prefeeding as gut priming to promote a gradual transition between endogenous and exogenous feeding.

Study 4 generated novel insights regarding the ontogeny and physiology of the European eel larval feeding stage. The establishment of first feeding larval culture is particularly challenged by the fact that *Anguillid* larvae do not appear attracted to live feed, while inert diets based on elasmobranch eggs have proven to serve the purpose for Japanese eel. Here, the three diets formulated and tested, included spiny dogfish egg yolk as a key component. Diet 1 represented the simplest composition, while Diet 2 and 3 were modifications, where proteins of different molecular size were added. Overall, the diet containing the larger peptides led to first information about development beyond the first feeding stage. Although the diet appears suboptimal, it is the first time that eel larvae survived throughout and beyond the first feeding stage, sustaining vital body functions past the point of no return. Physiologically, the longest living larvae were characterized by upregulated expression of genes related to digestion, food intake and growth, and downregulated *ghrelin*, encoding the “hunger hormone”. Thus, the inclusion of more complex dietary proteins (as in Diet 3), but not hydrolysed peptides (as in Diet 2) seems to promote larval morphological and physiological development after the successful transition to exogenous feeding. Hence, the composition of this diet provides important insights into larval nutritional requirements that can be used for further improvement of feeds and feeding regimes. Similar to the gut priming studies, the leakage of nutrients from inert feeds is an issue for consideration when advancing rearing techniques in terms of water quality management.

Altogether, the combination of methods applied in this PhD project has provided novel insights into the biochemistry, resource utilization, developmental physiology and nutritional requirements of European eel larvae in culture, especially considering that the establishment of first feeding culture is at a pioneering state. Based on consecutive targeted experiments, larval survival and growth was incrementally improved and insights substantiated. These results paved the way for forthcoming feeding experiments, eventually enabling larval growth, survival and metamorphosis into glass eels. This, together with ongoing multi- and interdisciplinary research, will enable hatchery development for sustainable aquaculture and support conservation of the critically endangered European eel.

8. References

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Manuscripts

Study 1:

Type of hormonal treatment administered to induce vitellogenesis in European eel influences biochemical composition of eggs and yolk-sac larvae

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Abstract Egg biochemical composition is among the main factors affecting offspring quality and survival during the yolk-sac stage, when larvae depend exclusively on yolk nutrients. These nutrients are primarily embedded in the developing oocytes during vitellogenesis. In aquaculture, assisted reproduction procedures may be applied enabling gamete production. For the European eel (*Anguilla anguilla*), reproductive treatment involves administration of pituitary extracts from carp (CPE) or salmon (SPE) to induce and sustain vitellogenesis. In the present study, we compared the influence of CPE and SPE treatments on offspring quality and composition as well as nutrient utilization during the yolk-sac stage. Thus, dry weight, proximal composition (total lipid, total protein), free amino acids, and fatty acids were assessed in eggs and larvae throughout the yolk-sac stage, where body and oil-droplet area were measured to estimate growth rate, oil-droplet utilization, and oil-droplet utilization efficiency. The results showed that CPE females spawned eggs with higher lipid and free amino acid contents. However, SPE females produced more buoyant eggs with higher fertilization rate as well as larger larvae with more energy reserves (estimated as oil-droplet area). Overall, general patterns of nutrient utilization were detected, such as the amount of total lipid and monounsaturated fatty acids decreasing from the egg stage and throughout the yolk-sac larval stage. On the contrary, essential fatty acids and free amino acids were retained. Notably, towards the end of the yolk-sac stage, the proximal composition and biometry of surviving larvae, from both treatments, were similar.

Keywords Assisted reproduction · Pituitary extract · Egg and larval quality · FAA · EFA · Lipids · Protein

Introduction

The production of good-quality eggs relies on the optimal progression of oogenesis, where the coordinated assembly of developing eggs is controlled by an interplay of endocrine and intra-ovarian factors, a process that can take a year or more (Tyler and Sumpter 1996). In vertebrates, pituitary gonadotropins regulate oogenesis through stimulation of sex steroid synthesis by follicle cells surrounding the developing oocytes (Nagahama and Yamashita 2008). The pituitary produces two types of gonadotropin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Brooks et al. 1997). In oviparous fishes,

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FSH acts on follicular granulosa and thecal cells, stimulating the synthesis of estradiol-17β (Suzuki et al. 1998), which among other stimulates the production of a glycophosphate lipoprotein (vitellogenin) by the liver (Lubzens et al. 2010; Li and Zhang 2017; Reading et al. 2018). Vitellogenin is transported to the ovary and incorporated into the developing oocytes as cytoplasmic yolk granules or globules (Ohkubo et al. 2008), while it is considered the major source of amino acids, lipids, and calcium necessary for embryonic development (Brooks et al. 1997). On the other side, the role of LH primarily relates to the final maturation of the follicle, stimulating the production of maturation-inducing hormone (MIH) and maturation-promoting factor (MPF), leading to ovulation (Nagahama and Yamashita 2008).

The composition of eggs is among the most recognized factors affecting offspring quality and survival, where the nutrient-bearing yolk portion has the primary function to nourish the developing embryos and larvae (Brooks et al. 1997; Bobe 2015). These nutrients, along with other cytological constituents, originate from maternal resources being deposited in the oocytes during the secondary growth phase (Reading et al. 2018). Overall, these patterns are similar in oviparous fishes; however, the exact composition of fish eggs is species-specific and the precise sequence of consumption and utilization by the developing embryo and larva varies.

Fish eggs can be classified into two energetic categories, i.e. without or with oil droplets, referred to Type I and Type II eggs (Rønnestad et al. 2013). Offspring of fish species with Type II eggs, including the European eel (*Anguilla anguilla*), acquire about half of their energy from amino acids and about half from lipids, in particular monounsaturated fatty acids (MUFAs). While MUFAs are utilized for energy generation (Rainuzzo et al. 1994; Finn et al. 1994; Sargent et al. 1999), yolk saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs) are retained to sustain development of the organism until the feeding stage (Kamler 2008). Thus, adequate amounts of PUFAs need to be present in the eggs (Bell and Sargent 2003), as suboptimal levels of essential fatty acids (EFAs), such as arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), can cause poor growth, skeleton deformities, and immune deficiency leading to increased mortality (Izquierdo 2005). Overall, the amount and composition of EFAs in the fish eggs and yolk-sac larvae is strongly influencing offspring quality (fertilization and hatching rate) and early larval survival (Tocher 2010).

Proteins are also fundamental constituents of fish eggs among other providing amino acids necessary for organ and muscle growth as well as dispensing energy via catabolic processes (Rønnestad et al. 1993). Thus, the yolk-sac comprises a pool of free amino acids (FAAs), representing around 50% of the amino acids present in marine fish eggs (Fyhn 1990). Already during final maturation of the oocyte, FAAs serve as osmotic effectors responsible for the influx of water and hydration that is required for neutral buoyancy of the eggs (Seoka et al. 2004). Later during yolk resorption, the FAA pool is depleted and reaches low levels at first feeding (Rønnestad et al. 1999). Thus, during the prefeeding period, larvae are highly dependent on the FAA pool as fuel in the energy dissipation, as shown for fish species such as lemon sole, *Microstomus kitt*, Atlantic halibut, *Hippoglossus hippoglossus* and cod, *Gadus morhua* (Rønnestad et al. 1993; Conceição and Tandler 2018).

In aquaculture, the control of gamete quality and successful offspring development is of immense importance (Migaud et al. 2013). Here, maternal dietary requirements must be met to obtain high-quality eggs, including adequate amounts of EFAs, in particular for marine fish that are incapable of synthesizing and prolonging PUFAs (Bell and Sargent 2003). Moreover, assisted reproduction procedures, including exogenous hormonal therapies, are often necessary to produce offspring in captivity (Bobe and Labbé 2010; Mylonas and Zohar 2010), which may influence oogenesis and egg quality. In the case of European eel, where closing the life cycle in captivity is targeted to sustain aquaculture, assisted reproduction protocols need to be applied to overcome a natural inhibition of sexual maturation in both sexes (Vidal et al. 2004; Tomkiewicz 2012; Mordenti et al. 2019). Female reproduction protocols involve repeated administration of pituitary extracts of carp (CPE) or salmon (SPE) to induce and sustain vitellogenesis, while administration of a maturation-inducing steroid is needed to complete oocyte maturation and ovulation (Palstra et al. 2005; Da Silva et al. 2018; Kottmann et al. 2021). Recently, Kottmann et al. (2020a), studying the differential impact

of CPE and SPE administration on egg quality and embryonic developmental competence, reported higher proportions of buoyant eggs and embryonic survival in offspring from SPE-treated females compared to CPE females. Thus, different content and composition of hormones in the pituitary glands and/or a species-specific affinity of eel receptors to carp and/or salmon hormones may influence egg quality (Schmitz et al. 2005).

In this context, we hypothesized that different hormonal therapies may influence oocyte development, resulting in variable egg biochemical composition and leading to differences in nutrient composition of eggs and yolk-sac larvae. As such, the objective of this study was to compare the effect of two maternal hormonal treatments (i.e. SPE vs. CPE) on the biochemical composition of European eel offspring and larval utilization of resources throughout early development. Fertilized eggs were sampled 4 h post-fertilization (hpf), while larvae were sampled at regular intervals from hatch to the first-feeding stage (at 0, 4, 8, and 13 days post-hatch (dph)). Biochemical variables measured included dry weight, total lipid, fatty acid, total protein, and free amino acid composition, while morphometric larval quality indicators included body area, oil-droplet area, growth rate, and oil-droplet absorption/utilization rate.

Material and Methods

Broodstock and gamete production

Wild-caught European eel females from a brackish lake (Saltbæk Vig, Denmark) were transported to the EEL-HATCH research facility of the Technical University of Denmark, located in Hirtshals, Denmark. Upon arrival, fish were randomly distributed at a density of ~ 15 fish/tank into replicated ~ 1150-L polyethylene tanks equipped with a recirculating aquaculture system (RAS), under a continuous flow rate of ~ 10–15 L min^{-1}, low-intensity light (~ 20 lx), and 12-h day:12-h night photoperiod (Tomkiewicz 2012). Male eels were obtained from a commercial eel farm (Royal Danish Fish) located in Hanstholm (Denmark) and reared in a similar, but smaller RAS (~ 450L capacity) at a density of 15–22 fish/tank under similar ambient conditions. During the experiment, water temperature and salinity were maintained at ~ 20 °C and ~ 36 psu, respectively, while feeding was ceased as eels naturally undergo a fasting period from the onset of the pre-pubertal silvering stage. Females were randomly divided into two groups. After two weeks of acclimatization, one group received weekly injections of salmon pituitary extract (SPE, 18.75 mg kg^{-1} initial body weight, Argent Chemical Laboratories, Washington, USA), while the second group received weekly injections of carp pituitary extract (CPE, same dose, and same supplier) to induce vitellogenesis (Kottmann et al. 2020a). For completion of final maturation and ovulation, an additional injection of PE was given in combination with administration of 17α,20ß-dihydroxy-4-pregnen-3-one (DHP, Sigma-Aldrich, St. Louis, MO, USA) at 2.0 mg kg^{-1} present body weight, with stripping of eggs 12–14 h after injection (Palstra et al. 2005; Kottmann et al. 2021). The male eels received weekly injections of human chorionic gonadotropin (hCG, Sigma-Aldrich, Missouri, USA) at 150 IU/fish (Pérez et al. 2000). An additional injection of hGC was given to male eels ~ 12 h prior to spawning (Koumpiadis et al. 2021). Stripped milt from four to five males per female (Benini et al. 2018) was pipetted into an immobilizing medium (P1 medium, Peñaranda et al. 2010) at a standardized concentration of 1:99 and used for fertilization within 4 h of collection (Butts et al. 2014; Sørensen et al. 2013).

Fertilization and embryonic incubation

Stripped eggs from each female were fertilized by a pool of standardized milt. Upon mixing of gametes, artificial sea water (ASW), i.e. tap water filtered through a reverse osmosis filtration system (Vertex Puratek 100 gpd RO/DI, Vertex Technologies Inc., CA, USA) salted up to 36 psu with Reef Salt (Red Sea, Red Sea International, Eilat, Israel), was added for activation followed by incubation at temperature of 20 °C (Sørensen et al. 2016a). Initially, the eggs were incubated in 15 L of ASW for 1 h, from where the buoyant egg layer was gently moved into new 15 L of ASW. Two hours post-fertilization (hpf), buoyant eggs were transferred to 60-L conical egg incubators and supplied with conditioned and filtered seawater at a flow through rate of ~ 350 mL min^{-1} (Politis et al. 2018b). Gentle aeration was added after ~ 10 hpf, while temperature was lowered to 18 °C for optimized embryonic development (Politis et al. 2017).

Light was kept at a low intensity of ~ 10 lx (Politis et al. 2014), and sinking dead eggs were purged from the bottom valve of each incubator. At ~ 52 hpf aeration was stopped and hatching occurred at ~ 56 hpf.

Larval culture

After hatch, each batch of larvae was transferred into separate 80-L tanks connected to a RAS system consisting of biofilter, trickle filter, UV (ProCristal UV-C 11 W, JBL GmbH & Co. Neuhofen, Germany), protein skimmer (AquaMedic 5000 single 6.0, Bissendorf, Germany), and reservoir for top-up. Temperature and salinity were maintained at 18 °C and 36 psu, respectively, with low-intensity illumination (Politis et al. 2014). On 4 dph, a fraction of each batch of larvae was transferred to three 8-L Kreisel tanks (~ 1000 larvae each tank). Each Kreisel was connected to a RAS, kept at 18 psu (Politis et al. 2018a; 2021) and 18 °C (Politis et al. 2017), while larvae were reared in total darkness and monitored under low-intensity illumination (Politis et al. 2014). Flow rates in tanks were kept at ~ 420 mL min^{-1}. The experiment continued until the larvae reached the first-feeding stage (13 dph).

Egg production, fertilization success, hatching success, and larval biometry

The weight of stripped eggs was recorded prior to fertilization, while ~ 30 min post-fertilization, the amount of buoyant eggs (%) was determined in a 25-mL volumetric column (Tomkiewicz 2012).

At 4 hpf, a sample of eggs (n ~ 100 eggs) was obtained and photographed using a stereomicroscope (SMZ1270i, Nikon Corporation, Japan) with a mounted camera (Digital Sight DS-Fi2, Nikon Corporation, Japan). Subsequently, digital images were analysed applying NIS-Elements-D analysis software (Version 3.2, Nikon Corporation, Japan). Using the 4-cell stage as criterion (Sørensen et al. 2016b), fertilization success was calculated as the percentage of fertilized eggs divided by the total number of eggs imaged. In order to estimate the embryonic survival at 24 and 48 hpf, the number of embryos was calculated in 3 × 15 ml water samples collected from the incubators. For the estimation of hatching success, subsamples of ~ 100 embryos were collected from the incubators at 48 hpf and inserted into 200-mL sterile tissue culture flasks (VWR, Denmark) filled with culture water enriched with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) (Sørensen et al. 2014). Approximately 12 h after hatching, the number of larvae and unhatched embryos was recorded to assess hatching success (%).

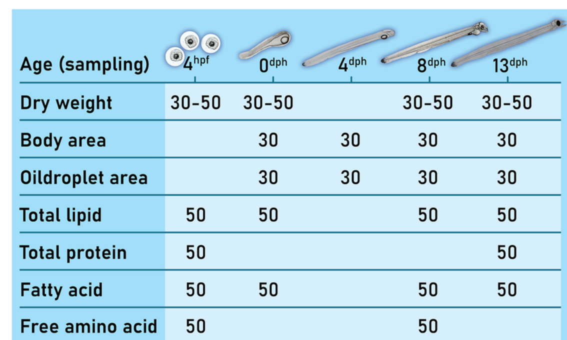
Sampling and data collection

The offspring from each of the 11 parental crosses were sampled at 4 hpf, at hatch and at regular intervals throughout the endogenous feeding larval stage until the first-feeding stage (0, 4, 8, 13 dph). Figure 1 provides a sampling overview and the number of eggs and larvae sampled for the various analyses.

For each batch, 10 larvae from each of 3 replicates were photographed at each sampling point (hatch, 0, 4, 8 and 13 dph) for biometric analyses. Body area (mm^2) and oil-droplet area (mm^2) were measured for each larva. Larval growth (GR) and oil-droplet utilization (ODU) were measured from the change in body and oil-droplet area from hatching (0 dph) until first feeding (13 dph) (Politis et al. 2017). Moreover, the oil-droplet utilization efficiency (ODUE) was calculated by dividing the increase in body area between 0 and 13 dph by the corresponding decrease of yolk area.

Dry weight and biochemical composition of eggs and larvae

Eggs and larvae were collected at 4 hpf and at 0, 8, and 13 dph, euthanized with MS-222, washed



Age (sampling)	4 hpf	0 dph	4 dph	8 dph	13 dph
Dry weight	30-50	30-50		30-50	30-50
Body area		30	30	30	30
Oildroplet area		30	30	30	30
Total lipid	50	50		50	50
Total protein	50				50
Fatty acid	50	50		50	50
Free amino acid	50			50	

Fig. 1 – Overview over measured variables included in the study and total number of specimens sampled and analysed per age and variable

in deionized water, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis (Christ Beta 2–15, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). After freeze-drying, samples were weighed using a microbalance (Mettler-Toledo MT5, Mettler-Toledo A/S, Denmark). For the analyses of total protein, FAAs, and lipids, samples were transported to the Centre of Marine Sciences (Faro, Portugal). Quantification of FA concentration was conducted at the Technical University of Denmark (Kgs. Lyngby, Denmark).

For lipid extraction of eggs (0.1 ml) and larvae (50 larvae per sample) a homogeneous mixture of chloroform, methanol, and distilled water (2:2:1.8) was used, following a modified method of Bligh and Dyer (1959). The lipid extracts were heated at 60 °C over night, and the lipid content was determined by gravimetry after evaporation of chloroform. Lipids were transferred and weighed on pre-weighed vials. Finally, the extracts were weighed on a Mettler Toledo MT5 scale (Mettler-Toledo A/S, Glostrup, Denmark; d = 0.1 µg) and the amount of total lipid was calculated as the percentage of dry weight (mg ind^{-1}).

The protein content of eggs and larvae was determined from 1 mg of freeze-dried samples and obtained by applying the Kjeldahl method with a conversion factor of 6.25 as described in AOAC (2006). Finally, protein content was calculated as a percentage of dry weight. Due to the small size of the samples, the analysis of protein content on 0 and 8 dph was not reliable and therefore not further considered in this study.

Lipids for estimation of FA composition were extracted from fertilized eggs and larvae at 0, 8, and 13 dph, following Folch et al. (1957). 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 auto-sampler 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 nine-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 FA methyl esters (FAME) was removed. The extraction was repeated twice, by the addition of 0.5 mL heptane. The combined sample was added to 2-mL screw top vials with silicone/PFTE septa and evaporated at 60 °C with additional nitrogen flow. Esterified samples were analysed by gas chromatography (Thermo Scientific Trace 1300), and analytes were detected by a single-quadrupole mass spectrometer (Thermo Scientific ISQ 7000). Split less injection was used and an inlet temperature of 220 °C. The carrier gas was helium and was set at a constant flow of 1.2 mL min^{-1} throughout the run. Separation of the FAME was achieved using a Thermo Scientific Trace GOLD TG-5MS column (length: 30 m; diameter: 0.25 mm; film thickness: 0.25 µm). The following temperature gradient was used for the separation: the initial temperature of 60 °C was held for 1 min, followed by an increase to 150 °C at 40 °C min^{-1}, and then slowly raised to 220 °C at 2.5 °C min^{-1}. The temperature was held at 220 °C for 14 min, raised to 300 °C at 40 °C min^{-1}, and finally held at 300 °C for 5 min. Ionization was undertaken by electron impact, and the mass range was set to 10–800 *m/z*. The MS transfer line was held at 250 °C, while the ion source temperature was set to 300 °C. FAME identification was undertaken by reference to an external standard consisting of a mixture of FAMEs (Supelco, F.A.M.E. Mix, C4-C24, 18,919-1AMP). Identification of samples was confirmed with the NIST library (similarity index threshold of 800). The FAME composition was determined as a proportion of the total integrated peak areas of all detected peaks. Detector linearity ($R^2 > 0.92$) over the observed ranges of FAMEs was confirmed by external calibration curves of each of the measured FAMEs of the standard mixture.

FAA content was determined in fertilized eggs and larvae at 8 dph. After homogenization of freeze-dried samples in 0.1 M HCl on ice, samples were centrifuged at 1,500 g at 4 °C for 15 min and supernatant deproteinized by centrifugal ultrafiltration (10-kDa cut-off, 2500 × g at 4 °C for 20 min). All samples were pre-column derived with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). All analyses were performed by ultra-high-performance liquid

chromatography (UPLC) on a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Instrument control, data acquisition, and processing were achieved using Waters Empower software.

Statistical analyses

Data from the experiment were analysed through a series of ANOVA models (Keppel 1991) using R Studio Software (Version 1.3.959, *RStudio: Integrated Development for R. RStudio, PBC, Boston, MA*). 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 log10 transformed. Alpha was set at 0.05. Tukey's analysis was used to compare least-squares means between treatments. Family ID (individual females and their offspring) was considered random in all models. When no significant interaction was detected for the tested dependent variable, the model was re-run with the interaction effect removed, analyzing main effects separately. Hence, we analysed the main effects of hormonal treatment (CPE and SPE) on offspring quality in terms of the various dependent variables (Table 1) in relation to stage of development (4 hpf and 0, 4, 8, or 13 dph). A series of pairwise t tests were used to calculate differences between groups (CPE vs. SPE), when only the effect of treatment was influencing the data.

Results

Reproductive success and offspring development

Table 1 presents data of the 11 females on their reproductive success in relation to hormonal treatment. Neither the length and initial weight of the females nor the amount of eggs produced per female were significantly different. However, the relative amount of buoyant eggs and relative fertilization success were significantly higher in SPE compared to CPE batches (for both $p < 0.01$), while embryonic survival and hatching success did not differ between the two groups.

Biometry

The body area of larvae increased from 2.2 ± 0.3 mm^2 at hatch (0 dph) to 4.3 ± 0.7 mm^2 at the end of the endogenous feeding stage (13 dph). The statistical analysis detected a treatment × age interaction ($p < 0.0001$). Thus, the model was decomposed to a series of reduced ANOVA models to determine the effect of treatment at each age (Fig. 2 A) and the effect of age for each treatment (Fig. 2 B and C). At hatch and 4 dph, the body area of SPE larvae was significantly larger than the body area of larvae from the CPE treatment ($p < 0.05$). This difference diminished with age, resulting in no significant difference between the larvae sampled from the two treatments at 8 and 13 dph ($p > 0.05$). Concomitantly, the oil-droplet area (mm^2) decreased from 0.942 ± 0.07 mm^2 at 0 dph to 0.0262 ± 0.015 mm^2 at 13 dph. Also here, the treatment × age interaction was significant ($p < 0.0001$), so the model was decomposed to determine the effect of treatment at each age (Fig. 2 D) and the effect of age for each treatment (Fig. 2 E and F). In particular, larvae from the SPE treatment had a significant ($p < 0.001$) larger oil-droplet area (0.993 ± 0.001 mm^2) at hatch compared to larvae of the same age from the CPE treatment (0.882 ± 0.001 mm^2). Later in development (at 4, 8, and 13 dph), this difference between offspring from the two treatment groups was no longer detectable ($p > 0.05$).

Growth rate, oil-droplet utilization rate, and oil-droplet utilization efficiency

Growth rate (mm^2/day), calculated over a period of 13 days, ranged between families from 0.021 ± 0.013 mm^2/day to 0.16 ± 0.006 mm^2/day and did not differ between treatment groups (Fig. 3 A). Oil-droplet utilization (mm^2/day) was significantly influenced by the maternal treatment ($p < 0.001$), with SPE batches having a significantly higher daily oil-droplet utilization than CPE ones (Fig. 3 B). However, oil-droplet utilization efficiency was not influenced by treatment (Fig. 3 C).

Dry weight, total lipids, and total protein

Dry weight showed no treatment × age interaction and was not influenced by treatment (Fig. 4 B), but varied during development ($p < 0.001$). Here, dry

Table 1 - Female data and reproductive success in relation to hormonal treatments (CPE vs SPE). Mean values (± standard deviation) for each group for all the characteristics of European eel broodstock, eggs, and embryos are also shown. * represent significant differences ($p<0.05$)

Female ID	Treatment	Length (cm)	Weight (g)	Injection (n)	Batch (g)	Buoyant eggs (%)	Fertilization rate (%)	Survival 24 hpf (%)	Survival 48 hpf (%)	Hatching rate (%)
D01D	CPE	74	720	10	1006	50	91.3	72.8	72.5	72.5
CDC6	CPE	65	458	14	9000	99	56.1	NA	42,7	39.1
CAFB	CPE	75	836	14	751	100	45.3	19.9	12.0	9.3
D1DB	CPE	83	1230	15	813	80	38.7	33.5	23.8	15.9
D08B	CPE	72	682	16	616	80	58.2	58.2	27.2	3.0
Mean CPE		73.9 (±6.3)	785.2 (±283.9)	13.8 (±2.3)	817 (±147.6)	81.8 (±20)	57.9 (±20.3)	46.13 (±23.8)	34.84 (±23.7)	27.93 (±28.4)
CBD2	SPE	55.5	320	17	763	90	80.9	53.6	29.1	7.9
DCB9	SPE	72	832	11	817	100	92.7	70.3	47.5	43.9
C96C	SPE	67	626	11	780	90	74.1	67.9	40.9	10.7
CB1A	SPE	72	790	12	780	95	67.9	61.8	35.6	33.4
CF9A	SPE	77	880	12	523	50	49.2	45.5	13.9	7.2
CBAF	SPE	71	784	12	791	90	36.9	32.2	14.6	13.3
Mean SPE		69 (±7.4)	705.29 (±207.2)	12.50 (±2.3)	742 (±108.9)	85.80* (±18)	66.95* (±20.6)	55.27 (±14.7)	30.24 (±13.8)	19.39 (±15.43)

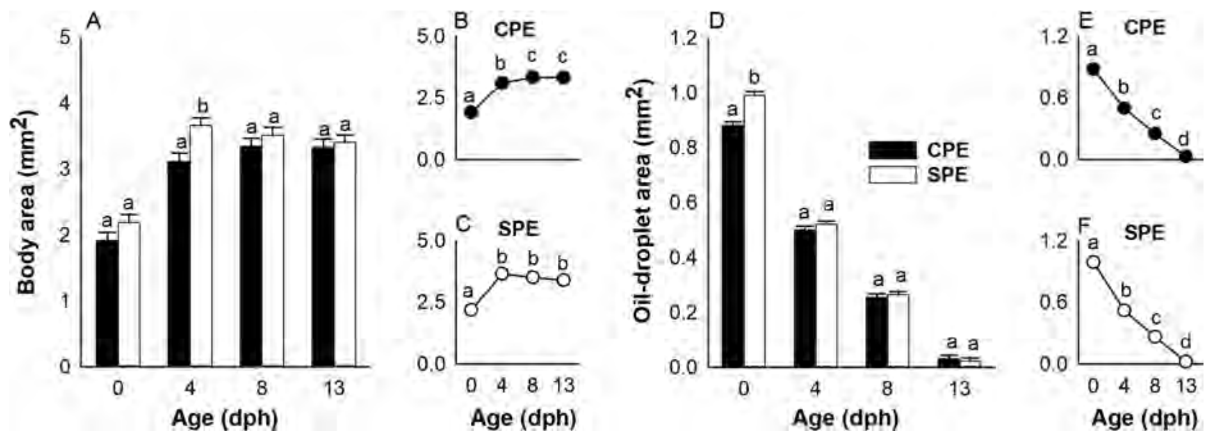


Fig. 2 – Association between maternal hormonal treatment (CPE vs SPE), body area (A, B, and C), and oil-droplet area (D, E, and F) of European eel larvae. Values represent means (±SEM) among batches. Different letters indicate significant differences ($p < 0.05$)

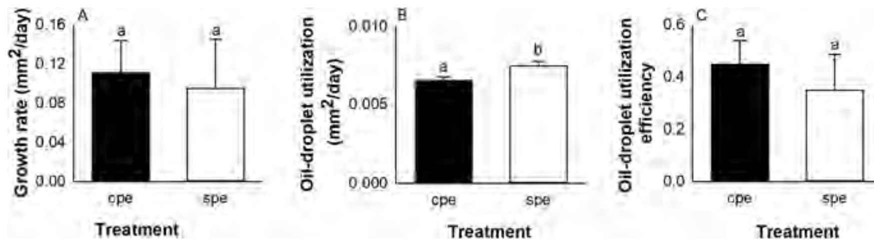


Fig. 3 – Effect of maternal hormonal treatment (CPE vs SPE) on growth rate (A), oil-droplet utilization (B), and oil-droplet utilization efficiency (C) in European eel larvae between hatch and the end of the endogenous feeding stage (13 dph). Values represent means (±SEM) among batches. Treatments with different letters are significantly different ($p < 0.05$)

weight significantly ($p < 0.0001$) reduced by ~ 50% from 0.045 ± 0.009 mg/individual at the egg stage to 0.026 ± 0.003 mg/individual at hatch. After hatch, dry weight of larvae gradually increased reaching 0.032 ± 0.007 mg/individual at 8 dph and 0.036 ± 0.009 mg/individual at 13 dph. No significant difference was observed between 0 and 8 dph nor between 8 and 13 dph (Fig. 4 A).

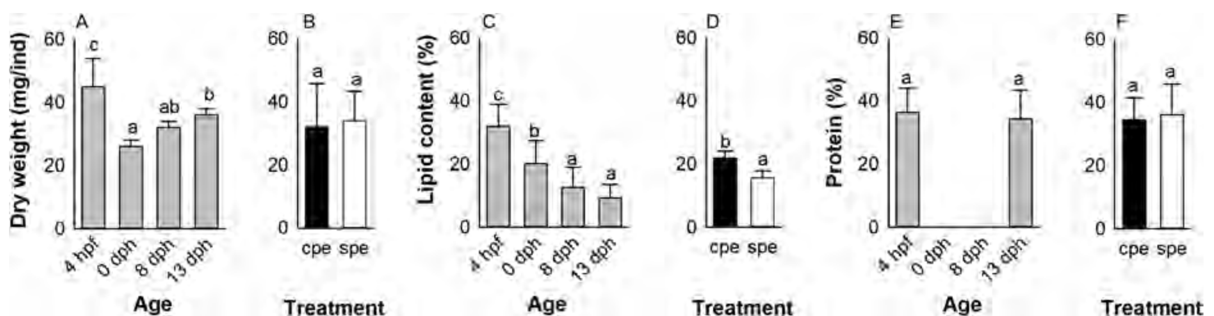


Fig. 4 – Effect of maternal hormonal treatment (CPE vs SPE) and age on dry weight (A, B), total lipid (C,D), and total protein (E,F) in eggs and larvae of European eel. Values represent means (±SEM) among batches. Different letters represent significant differences ($p < 0.05$)

On the other hand, total lipid content in offspring was significantly affected by maternal treatment ($p = 0.0071$) and age ($p < 0.0001$), while no significant treatment × age interaction was found. Total lipid content decreased from 37.62 ± 2.5% in fertilized eggs to 6.94 ± 2.3% in 13-day-old larvae. The total lipid content was significantly ($p < 0.01$) different between the egg stage and larvae at hatch as well as between hatch and 8 dph, while no significant difference was observed between 8 and 13 dph (Fig. 4 C and D). Moreover, CPE eggs and larvae contained more lipids (21.78 ± 2.121%) than their SPE counterparts (15.84 ± 2.324%).

In addition, total protein content measured in fertilized eggs and first-feeding larvae (13 dph) was not affected by treatment or age and the overall total protein content was stable around 35% throughout the endogenous stage (Fig. 4 E).

Free amino acids (FAAs) analysis

FAA content was analysed in fertilized eggs (4 hpf) and in larvae at 8 dph (Table 2). The FAA pool was divided into essential (EAA) and non-essential amino acids (NEAAs). The sum of EAAs increased significantly with age ($p < 0.001$), but was not affected by treatment ($p = 0.364$). EAAs were significantly affected by the treatment × age interaction ($p < 0.01$), including histidine, isoleucine, leucine, phenylalanine, threonine, and valine. In order to evaluate the effect of treatment at each stage, a series of one-way ANOVAs were performed. A significant difference in EAAs between treatments was present at the fertilized egg stage ($p < 0.001$), while this difference was no longer detectable in larvae at 8 dph ($p > 0.05$). On the other hand, levels of arginine, lysine, and methionine were significantly ($p < 0.01$) affected by age, but not by treatment. Moreover, tryptophan showed

Table 2 – Free amino acid values (± SD), essential in fertilized eggs, and yolk-sac larvae at 8 dph for two hormonal treatments (CPE and SPE) in European eel. Different lower and upper case letters represent significant statistical difference ($p < 0.05$)

	Fertilized eggs (4 hpf)		*Larvae (8 dph)*		*Model on fertilized eggs and larvae*		
	CPE	*SPE*	*CPE*	*SPE*	*Age x Treatment p-value*	*Age p-value*	*Treatment p-value*
Arginine	16.64 ± 4.76	14.63 ± 3.32	32.79 ± 2.66	34.34 ± 2.40	0.0755	< 0.00001	0.65311
Histidine	3.76 ± 1.11 [a]	1.44 ± 0.24 [b]	7.00 ± 0.77 [a]	7.01 ± 0.51 [a]	0.00118	-	-
Isoleucine	1.68 ± 0.15 [a]	2.95 ± 0.23 [b]	4.69 ± 0.51 [a]	5.07 ± 0.38 [a]	0.0074	-	-
Leucine	3.17 ± 0.33 [a]	4.71 ± 0.46 [b]	3.8 ± 0.43 [a]	3.88 ± 0.25 [a]	0.00026	-	-
Lysine	3.95 ± 0.41	4.99 ± 1.47	7.15 ± 0.84	7.52 ± 0.57	0.41078	< 0.0001	0.09394
Methionine	1.21 ± 0.14	1.30 ± 0.32	1.82 ± 0.24	1.82 ± 0.19	0.67026	< 0.00001	0.6115
Phenylalanine	2.33 ± 0.23 [a]	1.5 ± 0.35 [b]	4.65 ± 0.58 [a]	4.9 ± 0.38 [a]	0.00575	-	-
Threonine	2.69 ± 1.20 [a]	5.96 ± 2.18 [b]	12.9 ± 1.42 [a]	12.6 ± 0.78 [a]	0.01272	-	-
Valine	2.40 ± 0.37 [a]	0.54 ± 0.05 [b]	0.77 ± 0.06 [a]	0.86 ± 0.07 [a]	< 0.00001	-	-
Tryptophan	1.43 ± 0.027 [a]	1.56 ± 0.15 [b]	2.61 ± 0.23 [a]	2.92 ± 0.24 [b]	0.37075	< 0.00001	0.03068
ΣEAA	**39.25 ± 7.21**	**39.58 ± 4.62**	**76.31 ± 7.66**	**80.92 ± 5.61**	0.43476	< 0.00001	0.36415
Alanine	6.39 ± 0.98 [a]	1.46 ± 0.12 [b]	2.39 ± 0.22 [a]	2.43 ± 0.17 [a]	< 0.00001	-	-
Asparagine	3.03 ± 0.93 [a]	1.51 ± 0.60 [b]	4.71 ± 0.28 [a]	4.93 ± 0.37 [a]	0.00392	-	-
Aspartic Acid	1.80 ± 0.28 [a]	2.18 ± 0.21 [b]	3.44 ± 0.11 [a]	3.65 ± 0.24 [b]	0.33914	< 0.00001	0.04164
Cysteine	0.24 ± 0.02 [a]	0.34 ± 0.07 [b]	0.32 ± 0.02 [a]	0.32 ± 0.02 [a]	0.00981	-	-
Glutamine	20.61 ± 5.34 [a]	6.26 ± 0.92 [b]	33.23 ± 1.45 [a]	35.55 ± 2.68 a	< 0.00001	-	-
Glutamic acid	4.56 ± 0.75 [a]	6.27 ± 1.38 [b]	5.3 ± 0.32 [a]	6.30 ± 0.51 [a]	0.08054	0.11181	0.03583
Glycine	0.85 ± 0.23 [a]	0.96 ± 0.14 [b]	1.44 ± 0.06 [a]	1.59 ± 0.11 [a]	0.64644	< 0.00001	0.04943
Proline	4.77 ± 0.82 [a]	1.43 ± 0.13 [b]	3.27 ± 0.36 [a]	3.48 ± 0.29 [a]	< 0.00001	-	-
Serine	1.33 ± 0.34 [a]	3.08 ± 1.53 [b]	2.23 ± 0.22 [a]	2.34 ± 0.11 [b]	0.03431	-	-
Tyrosine	0.59 ± 0.1 [a]	0.88 ± 0.17 [b]	1.33 ± 0.13 [a]	1.44 ± 0.11 [b]	0.15918	< 0.00001	0.00568
ΣNEAA	**44.17 ± 8.78 [a]**	**24.38 ± 2.27 [b]**	**58.44 ± 5.66 [a]**	**62.02 ± 4.65 [a]**	0.00013	**-**	**-**
EAA/NEAA	0.89 ± 0.09 [a]	1.64 ± 0.18 [b]	1.31 ± 0.01 [a]	1.31 ± 0.01 [a]	< 0.00001	**-**	**-**
ΣFAA	**95.12 ± 5.43 [a]**	**70.28 ± 4.95 [b]**	**141.52 ± 5.43 [a]**	**150.05 ± 4.95 [a]**	0.00483	**-**	**-**

no treatment×age interaction, but was influenced by both, treatment ($p = 0.03$) and age ($p < 0.001$). Here, the amount of tryptophan increased with age and SPE eggs and larvae contained more tryptophan than CPE ones.

The sum of NEAA was significantly influenced by the treatment×age interaction ($p < 0.0001$). When this model was broken down to evaluate the effect of treatment at each age, a significant difference was evident between treatments at the fertilized egg stage, where eggs from CPE batches had almost the double amount of NEAA compared to SPE batches, while no difference was observed in larvae at 8 dph. Similarly, among the NEAA, alanine, asparagine, cysteine, glutamine, proline, and serine were also significantly affected by the treatment×age interaction; thus, the model was decomposed to test the effect of treatment at each stage. A series of one-way ANOVAs were performed for these EAA, also revealing a significant difference between treatments at the fertilized egg stage ($p < 0.001$), but no difference at 8 dph. Aspartic acid, glycine, and tyrosine were influenced by both treatment ($p < 0.0001$) and age ($p < 0.05$). These NEAAs increased with age and were higher in SPE eggs and 8 dph larvae compared to CPE. Glutamic acid was the only FAA influenced exclusively by treatment ($p = 0.035$).

The ratio EAA/NEAA was influenced by the treatment×age interaction ($p < 0.0001$). Once more, the model was broken down to evaluate the effect of maternal treatment at each age, showing that the ratio was significantly different among treatment groups at the egg stage ($p < 0.00001$), but not for 8 dph larvae.

Fatty acid analysis

The fatty acid composition in fertilized eggs (4 hpf) and developing yolk-sac larvae is presented in Table 3. The highest represented group was MUFAs,

Table 3 – Mean (±SD) proportion of main fatty acids, calculated as a percentage of total lipid in European eel fertilized eggs (4 hpf) and larvae (0, 8, and 13 dph)

Fatty acids	Egg	0 dph	8 dph	13 dph
C14:0	2.88 ± 0.54 [c]	2.01 ± 0.78 [b]	1.50 ± 0.23 [b]	1.11 ± 0.19 [a]
C15:0	0.33 ± 0.11	0.23 ± 0.14	0.25 ± 0.08	0.27 ± 0.04
C16:0	19.72 ± 1.69	22.54 ± 3.67	21.67 ± 3.18	25.26 ± 4.00
C17:0	0.57 ± 0.11	0.52 ± 0.11	0.51 ± 0.06	0.57 ± 0.07
C18:0	4.63 ± 0.40 [a]	6.19 ± 2.00 [b]	4.95 ± 0.48 [a]	7.40 ± 0.63 [c]
C20:0	0.21 ± 0.07 [ab]	0.18 ± 0.13 [a]	0.28 ± 0.09 [ab]	0.50 ± 0.12 [b]
ΣSFA	**28.03 ± 1.44 [ab]**	**31.43 ± 4.32 [ab]**	**29.15 ± 3.46 [a]**	**34.92 ± 4.33 [b]**
C16:1	8.93 ± 0.82 [d]	7.93 ± 1.28 [c]	6.41 ± 0.92 [b]	4.79 ± 0.74 [a]
C17:1	0.95 ± 0.17[c]	0.76 ± 0.23 [b]	0.75 ± 0.09 [b]	0.62 ± 0.07 [a]
C18:1	34.21 ± 4.11[bc]	37.60 ± 3.63[bc]	32.33 ± 2.79 [b]	28.38 ± 3.84 [a]
C20:1	1.19 ± 0.23 [b]	1.04 ± 0.29 [b]	0.81 ± 0.18 [a]	0.74 ± 0.15 [a]
ΣMUFA	**45.28 ± 3.46 [c]**	**47.34 ± 3.57[c]**	**40.30 ± 6.03 [b]**	**34.53 ± 4.39 [a]**
C18:2	2.32 ± 0.53	2.62 ± 0.32	2.60 ± 0.25	2.39 ± 0.41
C18:3	0.11 ± 0.08	0.08 ± 0.05	0.08 ± 0.05	0.08 ± 0.05
C20:2	0.20 ± 0.10	0.14 ± 0.09	0.17 ± 0.06	0.17 ± 0.11
C20:3	0.22 ± 0.08	0.16 ± 0.11	0.23 ± 0.08	0.25 ± 0.12
ΣPUFA	**2.58 ± 1.09**	**2.34 ± 1.38**	**3.01 ± 0.34**	**2.82 ± 0.66**
C20:4 – ARA	1.93 ± 0.61 [a]	2.11 ± 0.83 [a]	3.86 ± 0.50 [b]	6.58 ± 1.42 [c]
C20:5 – EPA	1.46 ± 0.41 [a]	1.47 ± 0.54 [a]	1.88 ± 0.18 [b]	2.33 ± 0.35 [c]
C22:6—DHA	3.92 ± 1.06 [a]	3.67 ± 1.47 [a]	6.08 ± 1.50 [b]	8.56 ± 2.63 [c]
Σn-3	5.47 ± 1.34 [a]	5.20 ± 1.95 [a]	8.02 ± 1.46 [b]	10.94 ± 2.98 [c]
Σn-6	3.77 ± 0.81	4.09 ± 0.79	4.48 ± 0.30	4.72 ± 0.57
n-3/n-6	0.71 ± 0.15 [a]	0.89 ± 0.37 [a]	0.58 ± 0.13 [b]	0.46 ± 0.13 [c]
EPA/ARA	0.74 ± 0.58[d]	0.70 ± 0.76[c]	0.48 ± 0.84[b]	0.35 ± 0.76 [a]
DHA/EPA	2.76 ± 0.09 [ab]	2.52 ± 0.13 [a]	3.26 ± 0.05 [b]	3.61 ± 0.05 [bc]
ΣFA	**87.75 ± 4.41**	**85.03 ± 5.28**	**84.62 ± 5.32**	**89.77 ± 5.26**

SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids, PUFA: poly-unsaturated fatty acid, ARA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid. Different letters represent significant differences (p < 0.05)

followed by SFAs and PUFAs. For all the fatty acids analysed in this study, no effect of treatment × age interaction was detected. Among the SFAs, the C14:0, C18:0, and C20:0 levels were affected by age ($p < 0.01$). Here, C14:0 showed an overall decreasing trend, while the proportion of the other two fatty acids increased throughout development. The proportions of the other SFAs analysed in this study (C15:0, C16:0, and C17:0) remained constant during the experimental period. The sum of MUFAs was significantly influenced by age, showing a decreasing trend throughout development. The sum of PUFAs was neither influenced by treatment nor age and remained constant between the fertilized egg stage and the end of the endogenous feeding stage. Levels of the essential fatty acids, EPA, ARA, and DHA, showed a significantly ($p < 0.01$) increasing trend during development, while the sum of *n-3* fatty acids significantly ($p = 0.0012$) increased between hatch (0 dph) and 13 dph. The sum of *n-6* was not affected by age or treatment but remained constant throughout the experimental period. The ratio EPA/ARA was significantly affected by age ($p < 0.00001$), where it decreased with development, while the opposite trend was apparent for the ratio DHA/EPA. The sum of fatty acids (ΣFA) remained constant throughout the experimental time.

Discussion

The present study assessed differences in quality and biochemical composition of European eel offspring produced via two alternative female treatment protocols (CPE *vs.* SPE). On the one side, CPE females produced eggs containing a higher amount of total lipids and free amino acids, while on the other, SPE females produced eggs showing a higher percentage of buoyant eggs and fertilization rate. The latter indicates that other maternally derived components than nutrients, such as mRNAs and steroid hormones, present in the egg cytoplasm, might govern the essential processes during early embryogenesis, as also recently suggested for European eel (Kottmann et al. 2020a; 2021).

Moreover, larvae from SPE females were larger at hatch than CPE. Considering the "bigger is better hypothesis" (Bailey and Houde 1989), having a larger body area at hatch is generally considered an advantage and has been related to higher survival for example in European sardine, *Sardina pilchardus* (Garrido et al. 2015), and capelin, *Mallotus villosus* (Chambers et al. 1989). However, this initial advantage did not translate into benefits neither in body area at 13 dph nor growth rate throughout the experimental period. In fact, the oil-droplet utilization (ODU) was higher in the originally smaller CPE larvae with smaller oil droplets, indicating an efficient usage of energy reserves, as CPE and SPE larvae grew to similar sizes at the end of the yolk-sac stage. In this regard, the oil-droplet (and/or yolk) utilization efficiency seems to not only depend on environmental conditions such as temperature, salinity, and oxygen (Kamler 2008), but also maternally derived hormones, such as testosterone and cortisol, as shown in species such as damselfish, *Pomacentrus amboinensis* (McCormick 1999).

It is notable that most of the differences between treatments were observed at the egg stage and/or shortly after hatch, while they were no longer detectable during later larval development. At this point, larvae possessed similar total lipid and protein amounts, concentration of free amino acids as well as body and oil-droplet area. This progressive reduction of maternal hormonal treatment effects may be related to the shift from early ontogenetic regulation by maternal factors (present in the egg cytoplasm) towards the organisms own regulation pathways, a process called maternal-to-zygotic transition, commonly occurring after the mid-blastula stage (Lee et al. 2014). Kottmann et al. (2020b) compared offspring from farm-raised females with wild-caught females and found an increased mortality during maternal-to-zygotic transition of egg batches scoring low initial quality parameters. Thus, regardless of reproduction methods or female origin, only larvae endowed with adequate energy reserves and sufficient nutrients appeared to meet the stage-specific requirements, surviving, and developing successfully. Failure to meet these requirements causes morphological deformities (Gwak and Tanaka 2001; Kjørsvik et al. 1991) and leads to high mortality (Dou et al. 2002; Houde 1974).

The two experimental groups of larvae exhibited common paths of utilization of the lipid, protein, fatty acid, and free amino acid resources. Despite the high variability in offspring quality, the gradual decrease in the amounts of total lipid and MUFA available at different yolk-sac stages was similar and in line with observations for Japanese eel, *Anguilla*

japonica (Furuita et al. 2006). This indicates that lipids and MUFAs were the main sources of energy during embryogenesis and early development of larvae. Beyond 8 dph, the lipid and MUFA content remained constant, suggesting that larvae at this stage are genetically pre-programmed to explore and exploit exogenous sources of lipids and fatty acids to meet their energy requirements. In fact, eel larvae start the expression of genes related to the main digestive enzymes (amylase, lipase, and trypsin) between 4 and 8 dph, marking the onset of molecular ontogeny of the feeding mechanism and the inclination to receive and process exogenous feed (Politis et al. 2018b). Among MUFAs, the oleic acid (C18:1) was the fatty acid with the highest concentration. This fatty acid, together with palmitic acid, DHA and EPA are considered among the essential fatty acids (Izquierdo 1996). Moreover, oleic acid and the other MUFAs have been regarded as main sources of energy during early development of marine fish larvae (van der Meeren et al. 2008). While MUFAs are consumed during the yolk-sac stage, palmitic acid and the other SFAs were not used, but preserved at later developmental stages. In addition, the amount of PUFA remained unchanged with larval development, as observed also in other marine fish species, where this class of fatty acids is preferentially incorporated into structural lipids in larval tissue (Wiegand 1996). This included that DHA, EPA, and ARA, playing a vital role in the physiology of fish larvae, were retained during the yolk-sac period. This pattern has been described also for other marine larvae such as Atlantic halibut (Rønnestad et al. 1995), turbot, *Scophthalmus maximus* (Rainuzzo et al. 1994), Atlantic cod (van der Meeren et al. 1993), and gilthead seabream, *Sparus aurata* (Rodriguez et al. 1994). Since eel larvae (as most marine fish species) are incapable of de novo synthesis of these fatty acids, EFAs are usually the last class of fatty acids used as energy source (Bell and Sargent 2003).

The eggs of European eel contained approximately 35% of protein, which is similar to previous reports on European eel and other eel species such as Japanese, American, *Anguilla rostrata,* and short-finned, *Anguilla australis* eel (Heinsbroek et al. 2013). In American shad, *Alosa sapidissima* (Liu et al. 2018), brill, *Scophthalmus rhombus L.* (Cruzado et al. 2013), and cobia, *Rachycentron canadum* (Huang et al. 2021) as well as Japanese eel (Ohkubo et al. 2008), the concentration of protein after hatching has been reported to decrease due to high demand for energy, besides being used as building blocks during embryonic development. In the present study, the protein content of the fertilized eggs was similar to the content of 13-day-old larvae and thus was retained during the entire endogenous feeding period. We can speculate that European eel embryos and larvae utilize the yolk protein to grow and develop their body, transforming from being part of the yolk reserve to be part of the body, without being used for energetic purposes.

Moreover, FAAs slightly increased from 7–10% at the egg stage to 14–15% in larvae at 8 dph, levels which are similar to Japanese eel, but tenfold lower than Atlantic cod and threefold lower than turbot (Fyhn 1990). As for total FAAs, the free amino acid pool was maintained between the fertilized egg stage and the middle-end of the yolk-sac larval stage. This differed from previous studies on Japanese and European eel (Heinsbroek et al. 2013; Ohkubo et al 2008), where FAAs decreased most between hatch and 4 dph. On the other hand, the FAA profile of the eggs in the present study was similar to that of Japanese eel eggs, where glutamine (Gln) shows highest concentration, followed by arginine and alanine (Ohkubo et al. 2008). Glutamine is considered a NEAA, but it has proven to be a fundamental requirement for the synthesis of glycosaminoglycan (GAG) (Watford 2015), which is the basic material of the gelatinous matrix congrid eel, *Ariosoma balearicum* larvae are made of (Donnelly et al. 1995). As discussed by Ohkubo et al. (2008), non-essential amino acids could be synthetized or converted within the egg/larvae due to specific needs and the author reckoned that Gln could be used for protein synthesis or as an energy source. Considering that the amount of Gln increased at 8 dph, it is possible that Gln has been synthesized to assemble the gelatinous matrix, typical for the leptocephalus larvae of the anguilliform species. However, further investigations are necessary to clarify the amino acid modifications during yolk proteolysis in European eel.

In conclusion, female broodstock treated with either of the two hormonal treatments (SPE and CPE) were able to produce viable offspring. The SPE protocol provided the highest quality of eggs in terms of higher fertilization rate and size (body area) at hatch.

However, CPE females produced offspring containing higher amounts of lipids and FAAs, which could potentially be of benefit to larval fitness in case of adverse environmental conditions or prolonged starvation. Nevertheless, after hatching, the differences between treatments levelled-out and were no longer detectable among larvae surviving to the first-feeding stage. Furthermore, eel larvae utilized MUFAs and lipids as main sources of energy until day 8, when they need to start switching to exogenous feeding to meet energy demands and constituent requirements. On the contrary, EFAs were not spent, which likely is due to their importance in regulating immune and inflammatory responses as well as cellular metabolism. Similarly, also FAAs were retained, probably to be used throughout later development.

Statements and Declarations

Conflict of interest:

The authors declare no competing interests.

Ethics approval

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Directive 2010/63/EU). Eel experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015–15-0201–00,696). Submergence in an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg L^{-1}, Sigma-Aldrich, Germany) was applied to anaesthetize individual eels in relation to tagging (all fishes), ovarian biopsy, and stripping of gametes (females) and to euthanize individuals after stripping (females) or at the end of the experiment (males). For larvae, tricaine methanesulfonate (MS-222, 25 mg L^{-1}, Sigma-Aldrich, Germany) was used to anaesthetize larvae before photography and to euthanize sampled larvae before preservation.

Consent to participate: All authors consent to participate.

Consent for publication: All authors approved the submitted version of this manuscript.

Availability of data and material:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability:

Not applicable.

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Authors' contribution: JT, SS, and SP provided funding. SP, JT, EB, and SE designed the study. SS and SP designed the rearing system. EB conducted experimental work and collected samples. EB and SE performed biochemical analyses. EB and AN performed statistical analysis. EB wrote original draft. All authors contributed to interpretation, manuscript revision, and approved the submitted version.

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Study 2:

First assessment of prebiotics, probiotics, and synbiotics affecting survival, growth, and gene expression of European eel larvae

S.N., Politis, E., Benini, J.J., Miest, S., Engrola, S.R., Sørensen, E., Syropoulou, I.A.E, Butts, J., Tomkiewicz

Manuscript

First assessment of prebiotics, probiotics, and synbiotics affecting survival, growth, and gene expression of European eel larvae

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Abstract

Eel larvae have been previously observed to ingest exogenous food later than the developmental functionality of the actual feeding apparatus, demonstrating the necessity for an earlier and/or improved transition to exogenous feeding. In the present study, prebiotics [AgriMOS, Mannan-oligosaccharides, and β-(1,3 and 1,6)-poly-D-glucose], probiotics (Bactocell, *Pediococcus acidilactici*), and synbiotics (AgriMOS + Bactocell), where administered to European eel larvae during the endogenous (pre-)feeding stage. Results revealed increased mortality when eel larvae were pre-fed with synbiotics, while growth was impaired in connection to probiotics and synbiotics, which could potentially be related to the high load of organic matter due to the pioneering experimental introduction of gut-priming agents directly into the rearing water, probably affecting water quality and increasing the bacterial load in the larval tanks. The lack of treatment related molecular responses in immune (*c3*, *igm*, *il10*, *il1β*, *irf7*) and stress/repair (*hsp70*, *hsp90*) genes, indicated a still immature immuno-readiness, probably caused by the lag phase between maternally inherited protection and the gradual build-up of the larvae's own immune system. As such, water management strategies and rearing options need to be adapted for future pre-feeding and feeding regimes, to target optimized culture conditions and ensure the production of healthy offspring. Moreover, genes relating to digestion (*try*, *ctra*, *ctrb*, *tgl*, *amyl*), food intake (*cck*), and appetite (*ghrl*), were expressed at basal levels already on 4 days post hatch, which combined with phenotypic plasticity of the appetite-regulating ghrelin (*ghrl*) towards gut-priming agents, indicate a prospective adaptive capacity towards earlier maturation of the larval gut to enhance digestive capacity. However, the gut-priming regimes applied did not seem optimally adapted for eel larval culture. Therefore, further investigations are encouraged to identify more suited gut-priming and/or pre-feeding strategies to improve the health and performance of cultured eel offspring.

Key words: Fish, *Anguilla anguilla*, early life history, gut-priming, gene expression

Introduction

Catadromous *anguillid* eels have continental juvenile stages followed by oceanic reproductive and larval stages, where so called silver eels and larvae (leptocephali) travel thousands of kilometres to complete their life cycle (Tesch, 2003). For the European eel (*Anguilla anguilla*), knowledge about natural reproduction and spawning habitats in the Sargasso Sea is limited, while insights regarding ecophysiology and nutrition of their offspring is negligible. However, during recent years significant progress has been made towards closing the life cycle of European eel in culture, enabling a steady production of high-quality gametes, embryos, and yolk-sac (pre-leptocephalus) larvae (reviewed in Tomkiewicz et al., 2019). In particular, early life challenges and preferences in relation to light, salinity, and temperature have been addressed (Politis et al., 2014; 2017; 2021), but nevertheless, metamorphosis to the subsequent leptocephalus stage remains a challenge mainly due to bottlenecks throughout the first-feeding period (Butts et al., 2016; Politis et al., 2018b).

Generally, mortality and poor growth at the onset of exogenous feeding have been widely reported for marine larviculture (May, 1974), which is also the case for the closely-related Japanese eel, *A. japonica* (Okamura et al., 2009; 2014). Failure during the transition from endogenous to exogenous feeding has been attributed to inadequate nutrition, both in quantity and quality, where food deprivation and/or unsuccessful initiation of first-feeding, following the exhaustion of yolk reserves, results in irreversible starvation (point-of-no-return), compromising larval survival and well-being (Yúfera & Darias 2007; Yúfera, 2011). Moreover, overall sensitivity during early development (Sørensen et al., 2014) is directly linked to the initially immature immune system, which predominantly relies on innate immune responses, rendering larvae vulnerable to pathogens in a microbe-rich environment (Vadstein & Bergh 2013; Miest et al., 2019), such as the one encountered in hatchery conditions, especially considering the host-

microbe interactions during intense feeding periods. Therefore, application of appropriate culture conditions throughout offspring culture, with respect to both abiotic and biotic factors, is essential.

Fish nutrition does not only depend on the feed source and availability, but also on the digestive potential of the fish, which is under endocrine control and linked to underlying genetic mechanisms (Zambonino-Infante & Cahu 2001; Zambonino-Infante & Cahu 2007; Rønnestad et al., 2013). Additionally, feeding and digestion functionality also relies on the microbial communities inhabiting the gastrointestinal tract (Romero et al., 2014). In this regard, gut microbiota support several functions within the host concerning the regulation of ingestion and metabolism (reviewed in Egerton et al., 2018; Butt & Volkoff 2019), as well as immune defence (reviewed in Vadstein & Bergh 2013). However, the microbiome during early life history is rather limited, as the larval gastrointestinal tract will progressively be colonized by microbes originating from the egg epibiota, the rearing water, and the first feed (Egerton et al., 2018). As such, gut microbial steering, to prime and assist or support digestion and health, has become of great interest. Especially for eel, larvae were observed to ingest exogenous food later than the developmental functionality of the feeding apparatus (Butts et al., 2016), demonstrating the necessity for an earlier and/or improved transition to exogenous feeding. Moreover, the expression profiles of genes linked to larval eel food intake and digestion related processes, indicate the potential adaptive capacity towards an earlier maturation of the digestive function and pancreatic enzyme secretion (Politis et al., 2018b). This, in combination with the early drinking capability already at hatch (Ahn et al., 2015), may facilitate the so called "*gut-priming*" principal even before the onset of exogenous feeding.

In this regard, gut-priming can be performed by microbial steering through the use of probiotics and prebiotics (Akhter et al., 2015). Probiotics, defined as live microorganisms,

which when administered in adequate amounts, confer a health benefit to the host, have been observed to enhance feed utilization, digestive enzyme activity, disease resistance, and concurrently survival and growth of farmed species in a direct or indirect way (Vine et al., 2006; Tihn et al., 2008; Dimitroglou et al., 2011; Jahangiri and Esteban 2018). Here, lactic acid bacteria have been frequently employed due to their adaptive capacity and beneficial effect towards microbial control and stability (Romero et al., 2014). However, introduction of “alien” microbiota for microbial control and stability, often entails a continuous supply to maintain the induced effect and may in some instances pose biosecurity threats. Thus, prebiotics, which are indigestible substrates that are offered as carbon source for intestinal fermentative bacteria, have been alternatively proposed to tackle such issues (Dimitroglou et al., 2011). Their selectivity promotes beneficial microorganisms, which outcompete pathogens either *via* antagonism, or through the production of health-related metabolites (reviewed in Ringø et al., 2010). Several saccharides have been exploited as prebiotics (Ringø et al., 2016), with mannan-oligosaccharides being commonly recruited to stimulate growth performance and nutrient digestibility as well as immunoreadiness (Torrecillas et al., 2014; Hoseinifar et al., 2015). Nonetheless, the effect of both prebiotics and probiotics varies, depending highly on the product, dose, time of supplementation and way of administration (Torrecillas et al., 2014; Jahangiri and Esteban 2018). In addition, a mixture of prebiotic and probiotic supplements has also been suggested (Cerezuela et al., 2011), where the so-called synbiotics, act synergistically improving survival and growth, through supportive action, on induction of digestive secretions (Huynh et al., 2017).

As such, in the present study prebiotics [AgriMOS: Mannan-oligosaccharides (MOS) and β-(1,3 and 1,6)-poly-D-glucose], probiotics (Bactocell: *Pediococcus acidilactici*), and synbiotics (AgriMOS + Bactocell), where administered to European eel larvae during the endogenous (pre-)feeding stage, with the aim to promote gut-priming and support their

digestive potential. The objectives were to assess the applicability and suitability of those nutritional additives as gut-priming agents for this species and investigate their effects on larval survival, biometrics (body area, oil-droplet area, growth, oil-droplet utilization and efficiency) and the molecular ontogeny of appetite (*ghrl*), food intake (*cck*), and digestion (*try*, *ctr-a*, *ctr-b*, *tgl*, *amyl*) as well as stress/repair (*hsp70*, *hsp90*), and immune (*c3*, *igm*, *il1b*, *il10*, *irf7*) related processes, through gene expression.

Material and Methods

Ethics statement

All fish were handled in accordance to the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696). Briefly, adult eels were anesthetized using ethyl p-aminobenzoate (benzocaine) before tagging and handling. European eel larvae were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222).

Broodstock management

Female broodstock were wild-caught from Saltbækvig, Denmark (55°44'51.1"N 11°08'28.3"E), while all males raised from the glass eel stage at a commercial eel farm (Royal Danish Fish, Hanstholm, Denmark). After collection, broodstock were transferred to the EEL-HATCH facility (DTU Aqua, Hirtshals, Denmark), where they were maintained in ~1250 L polyethylene tanks integrated into a recirculating aquaculture system (RAS), under a continuous flow rate per tank of ~10-15 L min^{-1}, low intensity light (~20 lux) and 12 h light/12 h dark photoperiod. Acclimatization took place over three weeks, to reach a salinity of 36 psu and temperature of 20°C. As eels naturally undergo a fasting period from the onset of the pre-pubertal silvering stage, they were not fed during this period. Prior to experimentation, the broodstock were anaesthetized (ethyl p-aminobenzoate, 20 mg L^{-1}; Sigma-Aldrich Chemie, Steinheim, Germany), tagged with a passive integrated transponder, and length and weight recorded.

Gamete production and embryonic incubation

To induce vitellogenesis, female eels received weekly injections of salmon pituitary extract (Argent Chemical Laboratories, USA) at 18.75 mg/kg body weight (Kottmann et al., 2020). To stimulate follicular maturation and induce ovulation, female eels received an additional injection of 17α,20ß-dihydroxy-4-pregnen-3-one (Sigma-Aldrich, St. Louis, USA) at 2.0 mg/kg body weight (Ohta et al., 1996; Kottmann et al., 2020b). Then, within 12–14 h, eggs were strip-spawned. Males received weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich Chemie, Germany) at 150 IU/fish. Prior to fertilization, they were given an additional injection and milt was collected ~12 h thereafter (Koumpiadis et al., 2021). Milt was pipetted into an immobilizing medium at a concentration of 1:99 (Sørensen et al., 2016b) and used for fertilization within 4 h of collection (Butts et al., 2014).

Eggs from each female were "crossed" with a pool of milt from several males to create different ($n = 3$) family crosses (Benini et al., 2018). Eggs from each female were stripped into dry plastic containers and gametes were swirled together. Artificial seawater (~20°C), prepared by using reverse osmosis filtration (Vertex Puratek 100 gpd RO/DI, Vertex Technologies Inc., USA) and salted to 36 psu (Aquaforest Reef Salt, Poland), was added for a gamete contact time of 5 min (Butts et al., 2014; Sørensen et al., 2016b). The fertilized eggs were then incubated for 2 h in 20 L containers filled with 15 L of the above-described artificial seawater before the buoyant eggs were transferred to 60 L black conical incubators, supplied with conditioned filtered seawater (Politis et al., 2018b) at a flow through rate of ∼350 mL/min. Gentle aeration was added after ∼4 hours post fertilization (hpf), while temperature was lowered to ~18°C for better embryonic development (Politis et al., 2017). Light was kept at a low intensity below ∼10 lux (Politis et al., 2014) and sinking dead eggs were purged from the bottom valve of each incubator in regular intervals. At ∼48 hpf, aeration was stopped, and larvae hatched at ∼56 hpf.

Experimental design and conditions

The experiment was repeated 3 times, each time using a different family cross (in total: *4 treatments* × *3 family crosses* × *3 replicates = 36 experimental units*). For each family cross (*n* = 3), at two days post hatching (dph), larvae were gently transferred into 24 acrylic jars (2 L Brine shrimp jars, Ø 13 cm, Height: 30 cm, Jug Desk-Type, Taipei, Taiwan), featuring bottom inlets with flow rates of ~150 mL / min and a 250 μm mesh subsurface outlet. Here, initial stocking density was ~200 larvae / L. The rearing jars were randomly divided into four groups (4 treatments × 3 replicates) and connected to four RAS units, each representing the 4 treatments (pre-, pro-, synbiotics, and control).

Table 1 - Commercial name, product description and amount of the pre-, pro- and synbiotics added daily to the rearing water of European eel, *Anguilla anguilla* larvae.

	Product name	Product description	Amount
Prebiotic	AgriMOS	*Mannan-oligosaccharides (MOS) and β-(1,3 and 1,6)-poly-D-glucose*	20g/m³/day
Probiotic	Bactocell	*Pediococcus acidilactici*	5g/m³/day
Synbiotic	Bactocell + AgriMOS		5g/m³/day + 20g/m³/day
Control	-	-	-

Each RAS unit consisted of a 50 L biofilter filled with RK bio-element, a protein skimmer (Wavereef, China), a 100 L reservoir hosting the main pump and a 180 L header tank. Three jars were connected to each system, while within each jar, a steady upwelling flow created enough turbulence to keep the larvae in suspension and maintained optimal oxygen levels for larval rearing. To keep the bacteria level under control, each system was connected to a UV-C lamp (11W JBL ProCristal UV-C, Compact, Germany), turned on from 9 pm to 9 am. Water temperature was kept at 19 ± 1°C and salinity was progressively reduced from 36 to 18 psu over a period of 4

days to improve larval survival (Politis et al., 2018a; Politis et al., 2021). Prebiotics (AgriMOS, Lallemand Inc, France), probiotics (Bactocell, Lallemand Inc, France), and synbiotics (Bactocell + AgriMOS) were daily added according to Lallemand Inc´s recommendations (**Table 1**) to the reservoir tank of the corresponding RAS (**Figure 1**). One RAS received no additives (control). Approximately 10% of water was exchanged daily.

Data collection

Survival

Considering each family cross (n = 3), treatment (n = 4) and replicate (n = 3), dead larvae were daily counted and removed from all experimental units. Additionally, all larvae at the end of the experiment as well as all sampled larvae from each experimental unit were enumerated and recorded. Larval cumulative mortality was then calculated as a percentage from 2 until 13 dph.

Biometry

At the beginning of each trial, ~15 larvae per family cross (n = 3) were randomly sampled, anesthetized with tricaine methanesulfonate (MS-222, Sigma-Aldrich, USA) and imaged using a digital camera (Digital Sight DS-Fi2, Nikon Corporation, Japan) attached to a zoom stereomicroscope (SMZ1270i, Nikon Corporation, Japan). Additionally, ~10 larvae from each family cross (n = 3), treatment (n = 4) and replicate (n = 3) were randomly sampled, anesthetized, and imaged at 4 (mouth opening), 8 (teeth formation), and 13 dph (end of the endogenous phase). NIS-Elements-D analysis software (Nikon Corporation, Japan) was used to analyze larval images, from where total body and oil-drop area were measured for each larva. Larval growth and oil-drop utilization rate were measured from the change in body and oil-drop area,

respectively. Growth efficiency was then measured by dividing the increase in body area by the corresponding decrease in oil-drop area (Politis et al., 2014).

Gene expression

For molecular analysis, ~30 larvae from each family cross (n = 3), treatment (n = 4), and replicate (n = 3) were randomly sampled at the beginning of the study (2 dph) and throughout the endogenous feeding stage (4, 8, and 13 dph). Those larvae were recorded, euthanized using MS-222, preserved in RNAlater Stabilization Reagent and kept at -20°C following the procedures described by Politis et al. (2021). RNA was extracted using the NucleoSpin RNA Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry using NanoDrop™ One (ThermoFisher Scientific™) and then transcribed using the qScript™ cDNA Synthesis Kit (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipe out step [PerfeCta DNase I Kit (Quantabio, Germany)]. The expression levels of target genes were determined by quantitative real-time PCR (RT-qPCR), using specific primers (**Table 2**), which were designed using primer 3 software v 0.4.0 (http://frodo.wi.mit.edu/primer3) based on cDNA sequences available in GenBank databases. All primers were designed for an amplification size ranging from 75 to 200 nucleotides and optimal Tm of ~60°C.

Expression of genes in each larval sample from each family cross (n = 3), treatment (n = 4), replicate (n = 2), and larval age (2, 4, 8, and 13 dph) were analysed in two technical replicates using the qPCR Biomark™ HD technology (Fluidigm) based on 96.96 dynamic arrays (GE chips). A pre-amplification step was performed with a 500 nM primer pool of all primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 µL cDNA per sample for 10 min at 95°C and then 14 cycles of 15 sec at 95°C, and 4 min at 60°C. Obtained PCR products were diluted 1:10 with low EDTA-TE buffer. The pre-

amplified 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. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60°C. The relative quantity of target gene transcripts was normalized (ΔCT) to the geometric mean of the 2 most stable reference (housekeeping) genes. The *ef1a* and *rps18* genes were chosen as housekeeping genes, after qBase+ software revealed that these mRNA levels were stable throughout analyzed samples ($M < 0.4$); M gives the gene stability and $M < 0.5$ is typical for stably expressed reference genes (Hellemans et al., 2007). Coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Table 2 - Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR. Primers were designed based on sequences available on Genbank databases.

Full Name	*Function*	*Abbrev.*	*Primer sequence (5' 3') Forward*	*Primer sequence (5' 3') Reverse*	*GenBank ID*
Elongation factor 1	Reference	*ef1a*	CTGAAGCCTGGTATGGTGGT	CATGGTGCATTTCCACAGAC	EU407824.1
18s ribosomal RNA		*rps18*	ACGAGGTTGAGAGAGTGGTG	TCAGCCTCTCCAGATCCTCT	XM_035428274.1
Cholecystokinin	Food intake	*cck*	CGCCAACCACAGAATAAAGG	ATTCGTATTCCTCGGCACTG	XM_035409023.1
Prepro-Ghrelin	Appetite	*ghrl*	TCACCATGACTGAGGAGCTG	TGGGACGCAGGGTTTTATGA	XM_035381207.1
Amylase	Digestion	*amyl2a*	AGACCAACAGCGGTGAAATC	TGCACGTTCAAGTCCAAGAG	XM_035420193.1
Triglyceride lipase		*tgl*	CTGACTGGGACAATGAGCGT	CGTCTCGGTGTCGATGTAGG	DQ493916.1
Chemo trypsin a		*ctra*	CCAGTAACGCTTGCAGTCAG	CCAGTAACGCTTGCAGTCAG	XM_035417209.1
Chemo trypsin b		*ctrb*	ATTGTTGATGGTGCTGGGGT	ATCGTGGGAGAGCATGACAA	XM_035417164.1
Trypsin		*try*	CTGCTACAAATCCCGTGTGG	GGAGTTGTATTTGGGGTGGC	MH001533.1
Heat shock protein 70	Cellular Stress/Repair	*hsp70*	TCAACCCAGATGAAGCAGTG	GCAGCAGATCCTGAACATTG	XM_035380750.1
Heat shock protein 90		*hsp90*	ACCATTGCCAAGTCAGGAAC	ACTGCTCATCGTCATTGTGC	XM_035392491.1
Complement system	Immune response	*c3b*	AATATGTGCTCCCAGCCTTC	GATAACTTGCCGTGATGTCG	XM_035404253.1
Immunoglobulin M		*Igm*	CCAAGGACCATTCTTTCGTC	ACTGGCTTTCAGGAAGATGC	EF062515.1
Interleukin 10		*Il10*	CCTGCAAGAAACCCTTTGAG	TGAACCAGGTGTCAATGCTC	XM_035382126.1
Interleukin 1β		*il1β*	ATTGGCTGGACTTGTGTTCC	CATGTGCATTAAAGCTGACCTG	XM_035380403.1
interferon regulatory factor 7		*Irf7*	TTCCTTGGAAGCACAACTCC	TGTCGTTCGGATTCTCTCTG	KF577784.1

Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., USA). Residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted valves) to ensure they met model assumptions. Data were $\log_{10}$ or arcsine square root (percentage data) transformed to meet these assumptions when necessary. Alpha was set at 0.05 for testing main effects and interactions. Treatment means were contrasted using Tukey's test. Body area, oil droplet area, growth rate, oil droplet utilization, growth efficiency, and survival at 13 dph as well as gene expression (14 genes) at each age (4, 8, and 13 dph) were analyzed using a series of mixed model factorial ANOVAs. The main model variables were gut-priming treatment (fixed effect), family (random effect), and the family × treatment interaction (random effect). Variance components (VC) for random effects were generated using the Restricted Maximum Likelihood estimation method and expressed as a percentage. The mean and standard error for each treatment and family effect were calculated. Additionally, a series of mixed effects models were run to investigate gene expression changes over time and throughout early larval ontogeny for each gut-priming regime (control, pre-, pro-, and synbiotics). These ANOVA models included the larval age (4, 8, 13 dph) fixed effect, the random family effect, and the random age × family interaction.

Results

Survival and biometry

Eel larval survival (until 13 dph) was not affected by the addition of pre- or probiotics and ranged from 52.7 ± 3.6 to 61.6 ± 4.6 % between treatments (pre, pro, and control), but was significantly reduced ($p < 0.01$) when larvae were pre-fed with synbiotics (34.3 ± 4.18 %). Moreover, a significant ($p < 0.01$) effect of family cross was observed, explaining 38.6 % of the total variance (Figure 2 A).

Larval body area did not differ across treatments, reaching 3.61 ± 0.02 mm^2 on 13 dph, where a significant treatment × family interaction explained 36.2 % of the total variance (Figure 2 B). Similarly, oil droplet area was not affected by treatments, reaching 0.0098 ± 0.00029 mm^2 on 13 dph, while a significant ($p < 0.01$) effect of family cross explained 95.5 % of the total variance (Figure 2 C). Moreover, growth rate was highest when eel larvae were pre-fed with prebiotics (0.062 ± 0.005 mm^2/d) or reared without gut-priming agents in the control treatment (0.069 ± 0.007 mm^2/d), but significantly ($p < 0.001$) reduced when pre-fed with probiotics (0.037 ± 0.005 mm^2) and synbiotics (0.035 ± 0.004 mm^2/d) (Figure 2 D). Here, the significant ($p < 0.01$) treatment × family interaction, explained 38.8 % of the total variance. At the same time, no statistically significant difference in energy reserve (oil droplet) utilization rate (0.007 ± 0.0003 mm^2/d) was observed, where the VCs for family cross explained 90.4 % of the observed variance (Figure 2 E). Similarly, no statistically significant difference in growth efficiency (0.215 ± 0.013 mm^2/d) was observed among treatments, where VCs for family cross and treatment × family interaction explained 45.1 % and 34.2 % of the total variance, respectively (Figure 2 F).

Appetite, food intake and digestion related gene expression

The expression of ghrelin (*ghrl*) significantly ($p < 0.01$) increased throughout development peaking at 13 dph (Figure 3 A), where it was driven by the significant ($p < 0.01$) family × treatment interaction, explaining 48.7 % of the variability (Figure 3 B). Similarly, the expression of cholecystokinin (*cck*) also significantly ($p < 0.01$) increased throughout development (Figure 3 C), while 53.9 % and 38.6 % of the observed variability on 4 and 8 dph, respectively, was driven by the significant ($p < 0.01$) family effect (Figure 3 D).

All genes investigated in this study, encoding major digestive enzymes, such as trypsin (*try*) and chymotrypsin homologs (*ctr-a* and *ctr-b)* as well as triglyceride lipase (*tgl*) and amylase (*amyl*), showed similar expression patterns, where a significant ($p < 0.01$) increase throughout development was observed, peaking at 13 dph (Figure 3 E-I). The VC analysis revealed a significant ($p < 0.01$) family effect, explaining the variability observed for *try* (75.1 %) and *ctr-a* (91.6 %) on 4 dph, *ctr-a* (56.8 %)*, ctr-b* (90.9 %) and *tgl* (47.6 %) on 8 dph as well as *ctr-a* (29.7 %)*, ctr-b* (43.1 %)*, tgl* (72.1 %) and *amyl* (64.3 %) on 13 dph (Figure 3 J-N).

Stress/repair and immune response related gene expression

Regarding immune response, the complement component (*c3*), immunoglobulin (*igm*), and interleukin 10 (*il10*) expression significantly ($p < 0.01$) decreased, while interleukin 1 β (*il1β*) and interferon regulating factor 7 (*irf7*) significantly ($p < 0.01$) increased throughout development (Figure 4 A-E). At the same time, the VC Analysis revealed a family effect, significantly ($p < 0.01$) explaining the variability observed for *irf7* (44.5 %) on 4 dph, for *igm* (41.8 %) and *il1β* (36.5 %) on 8 dph as well as for *il10* (14.9 %) on 13 dph (Figure 4 F-J).

Regarding stress/repair response, the expression of two genes encoding different types of heat shock proteins (*hsp70* and *hsp90*) significantly ($p < 0.01$) increased throughout development (Figure 4 K-L), while no significant effect of treatments was detected (Figure 4 M-N).

Discussion

Eel larvae have been previously observed to ingest exogenous food later than the developmental functionality of the actual feeding apparatus (Butts et al., 2016), demonstrating the necessity for an earlier and/or improved transition to exogenous feeding. As such, the present study examined the influences of prebiotics (AgriMOS), probiotics (Bactocell), and synbiotics (Bactocell + AgriMOS) on European eel larval survival, biometrics, and gene expression, hypothesizing that they would act as gut-priming agents, promoting the maturation of the larval gut and possibly stimulating the larval digestive capacity. The choice of gut-priming agents was based on positive findings in other fish species, but in this study their application directly into the rearing water was pioneeringly tested. For instance, the administration of lactic acid bacteria, similar to the ones present in Bactocell, improved growth, immunity and health status as well as reproduction in zebrafish, *Danio rerio* (Ahmadifar et al., 2020; Gioacchini et al., 2010), increased survival rates in pikeperch, *Sander lucioperca* larvae (Yanes-Roca et al., 2020), improved stress resistance in Persian sturgeon, *Acipenser persicus* larvae (Taridashti et al., 2017), and induced higher activity of digestive enzymes in juvenile California halibut, *Paralichthys californicus* (Zacarias-Soto et al., 2011). Similarly, the use of oligosaccharides, similar to the ones present in AgriMOS, have improved the growth performance, survival, and immune status of rainbow trout, *Oncorhynchus mykiss* (Staykov et al., 2007) or enhanced growth performance and feed utilization of the giant freshwater prawn, *Macrobrachium rosenbergii* juveniles (Mohamed et al., 2017).

We observed that the presence of gut priming agents can influence eel larval survival, as evident by the administration of synbiotics. Moreover, growth rate was also negatively affected by the administration of synbiotics or probiotics alone, while larvae receiving only prebiotics showed similar growth rates to larvae reared without gut-priming agents (control). In this study, eel larvae were reared in tanks connected to separate RAS for each treatment, which included a biofilter and UV treatment, targeting stability by digestion of waste nutrients and reduction of circulated pelagic microorganisms. However, it is worth mentioning, that generally the load of bacteria in a newly started RAS is normally much higher than in flow through systems or microbially matured systems (Vadstein et al., 2018). Therefore, when introducing microbiota (probiotics) and oligosaccharides (prebiotics), a stimulation of microbial growth and activity in the rearing water, but also the host, is expected. In this regard, a non-matured RAS, could potentially be negatively influenced by excess microbial activity, often driven by heterotrophic bacteria (Rojas-Tirado et al., 2018), resulting in compromised water quality of the entire rearing unit. Moreover, high abundance of these bacteria may directly or indirectly affect the host organism, by supporting the rise of opportunistic pathogens or potentially affecting the nitrification process of the RAS as they compete for substrate and oxygen with the autotrophic bacteria (Blancheton et al., 2013; Michaud et al., 2014). Hence, the synbiotics administered to eel larvae in this study, which were the combined pre- and probiotics, where prebiotics acted as a promoter for extrapolated probiotic proliferation, have likely caused deterioration of water quality, resulting in the registered impaired growth and survival.

At the same time, the high mortality observed in the synbiotic treatment, can also be related to the previously described immunocompromised period between hatch and the first-feeding stage of eel larvae (Miest et al., 2019). We observed the initially upregulated mRNA levels of genes corresponding to maternally originating immune protection,

exerted by the complement component *c3*, immune globulin type M (*igm*), and interleukin-10 (*il-10*), to decrease throughout ontogeny, following the utilization of the maternal energy resources (yolk-sac and oil-droplet) and reaching baseline levels already at 8 dph. Similar patterns have been observed also in other fish species such as European seabass, *Dicentrarchus labrax* (Breuil, 1997) and carp, *Cyprinus carpio* (Huttenhuis et al., 2006). On the other hand, the expression of interleukin 1β (*il1β*), interferon regulating factor 7 (*irf7*) and heat shock proteins (*hsp70* and *hsp90*) increased steadily during ontogeny, indicating a maturing functionality of the own eel larval stress/repair and immune response potential. However, we did not observe a molecular response in connection to the gut-priming agents administered in this study, probably indicating a still immature immuno-readiness caused by the lag phase between the maternally inherited immune protection and the gradual build-up of the larval own immune system.

Moreover, in the present study, the expression of trypsin (*try*), chymotrypsin (*ctra* and *ctrb*), lipase (*tgl*), and amylase (*amyl*) increased throughout ontogeny to peak at 13 dph, confirming the molecular ontogenetic start of the first-feeding window in European eel pre-leptocephalus larvae. However, we did not register a molecular benefit on digestive capacity through the addition of the applied gut priming agents, which contrasts with previous findings, showing that gut priming has the potential to act as a trigger for early development and functionality of digestive enzymes (Merrifield et al., 2010; Ibrahem, 2015). In particular, probiotics have improved the activity of amylase and lipase in yellow fin tuna, *Thunnus albacares* fry (Haryanti et al., 2021) and promoted intestinal development, improving immune enzyme activities and modulating gut microbiota, in yellow croaker, *Larimichthys crocea* larvae (Yin et al., 2021). Similarly, prebiotics have enhanced survival of first feeding turbot, *Scophthalmus maximus* larvae by altering immunity, metabolism, and microbiota (Miest et al., 2016). As such, the lack of altered

molecular digestive capacity responses in the present study, indicates that the molecular ontogeny of the key players relating to the hydrolytic function of the digestive system, was genetically pre-programmed and not modified by the applied gut-priming scenarios, possibly demonstrating that the choice of pre- and probiotics needs to be reconsidered for eel larviculture.

In this regard, the choice of appropriate probiotic regime depends on the probiont species, target fish species, and physiological status as well as rearing conditions and the specific goal of the application (Merrifield et al., 2010). For instance, two commonly seen as pathogenic *Vibrio* strains showed positive effects on scallop, *Argopecten purpuratus* larvae (Riquelme et al., 1996), while *Pseudomonas aeruginosa*, a member of the pathogenic skin microflora, acts as probiotic for western king prawns, *Penaeus latisulcatus* (Hai et al., 2009). Similarly, the fish pathogen S*hewanella putrefaciens* (Austin & Austin, 2016), is used as probiotic in gilthead sea bream, *Sparus aurata* and Senegalese sole, *Solea senegalensis* (García de la Banda et al., 2012; Tapia-Paniagua et al., 2012). As such, bacterial strains that are commonly harmful to one aquatic species, can be beneficial to another species when used as probiotic (Hai, 2015). Therefore, the sub-strains or phylogenies need to be identified and carefully considered before application as probiotics for a specific target fish species. Consequently, we speculate that the gut-priming agents applied in this study are not suited for universal use or at least not optimally adapted for eel larval culture.

Interestingly, the VC analyses of this study showed that the “family” effect drove most gene expression patterns, which could be translated into genetically pre-programmed molecular mechanisms, endowed by the parents, to control early ontogenetic processes. However, for each genotype (family), phenotypic trait, and treatment, a different reaction norm can exist (Pfennig et al., 2010; Kelly et al., 2012; Jarquín et al., 2014). In fact, the expression of appetite-regulating ghrelin (*ghrl*) was driven by a genetic (family) ×

treatment interaction, revealing differing reaction norms of each genotype (family) used in our study to the different treatments (pre-, pro- or synbiotics) investigated. As such, this result documents phenotypic plasticity to gut-priming agents, as each genotype can produce different phenotypes (in terms of gene expression) when exposed to different additives (pre, pro- or synbiotics). This, from one side demonstrates the sensitivity for adaptation potential towards gut-priming, but from the other side indicates that the gut-priming regimes applied in this study cannot be considered a "*one size fits all*" solution. However, it is worth mentioning that transcripts of genes relating to digestion, food intake and appetite, were in the present study already detected at 4 dph, evidencing that those endocrine mechanisms are present at basal levels at this early stage, before progressively gaining functionality throughout ontogeny. This indicates a prospective adaptive capacity towards earlier maturation of the eel larval appetite, feeding and digestion potential. Therefore, further investigations are encouraged to identify more suited gut-priming and/or pre-feeding strategies for culturing eel larvae in the future.

To summarize, the increased mortality observed in connection with synbiotics and the impaired growth observed in connection with probiotics and synbiotics, could potentially be related to the high load of organic matter in the rearing water, which probably affected water quality and increased the bacterial load in the larval tanks. At the same time, the lack of molecular responses in immune and stress/repair related genes, indicate a still immature immuno-readiness, probably caused by the lag phase between the maternally inherited protection and the gradual build-up of the larval own immune system. As such, water management strategies and rearing options need to be adapted for future pre-feeding and feeding regimes, to target optimized culture conditions and ensure the production of healthy offspring. Moreover, the early presence of genes relating to digestion, food intake, and appetite, as evidenced by their basal transcript levels already on 4 dph, as well as the phenotypic plasticity of the appetite-regulating ghrelin (*ghrl*)

towards gut-priming agents, indicate a prospective adaptive capacity towards earlier maturation of the larval gut to enhance digestive capacity. However, despite the valuable knowledge gained by this study, the gut-priming regimes applied did not seem optimally adapted for eel larval culture. Therefore, further investigations are encouraged to identify more suited gut-priming and/or pre-feeding strategies for culturing eel larvae in the future. Specifically, elucidation of the eel larval intestinal microbiota and host-microbiota interactions could lead to the development of more refined and efficacious microbiota-intervention strategies to improve the health and performance of cultured eel offspring.

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Author's contribution

JT, SP and SS provided funding. SP, JT, EB and SE designed the study. SS and SP designed the rearing system. JT established the assisted broodstock protocols providing offspring for the experiment. SP, EB and ES conducted the experiment and collected samples. SP, EB and JM conducted gene expression analysis. IB and SP ran statistics and analyzed data. SP and EB made illustrations. SP and JM designed primers. SP, EB and ES wrote original manuscript draft. All authors contributed to data interpretation, manuscript revision and approved the submitted version.

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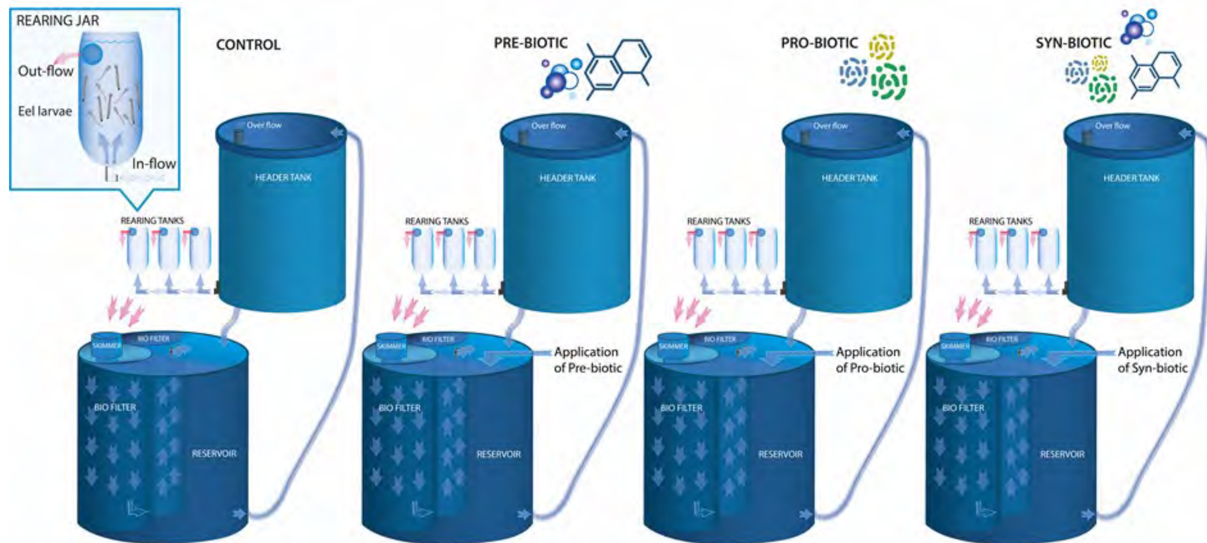


Figure 1: Schematic representation of the experimental setup, testing the application of prebiotics (AgriMOS), probiotics (Bactocell), and synbiotics (Bactocell + AgriMOS) as gut-priming agents for European eel, *Anguilla anguilla* larvae. Larvae were reared in replicated acrylic 2L jars, featuring bottom inlets with flow rates of ~150 mL / min and a 250 µm mesh subsurface outlet. Each treatment (control, pre-, pro-, and syn-biotics) was represented by three replicated jars connected to a separate recirculating aquaculture system (RAS). Each RAS unit consisted of a 50 L biofilter, protein skimmer, 100 L reservoir hosting the main pump and 180 L header tank. Prebiotics (20 g/m³), probiotics (5 g/m³), and synbiotics (prebiotics (20 g/m³) + probiotics (5 g/m³)) were daily added according to Lallemand Inc´s recommendations to the reservoir tank of the corresponding RAS. One RAS received no additives (control). Approximately 10% of water was exchanged daily.

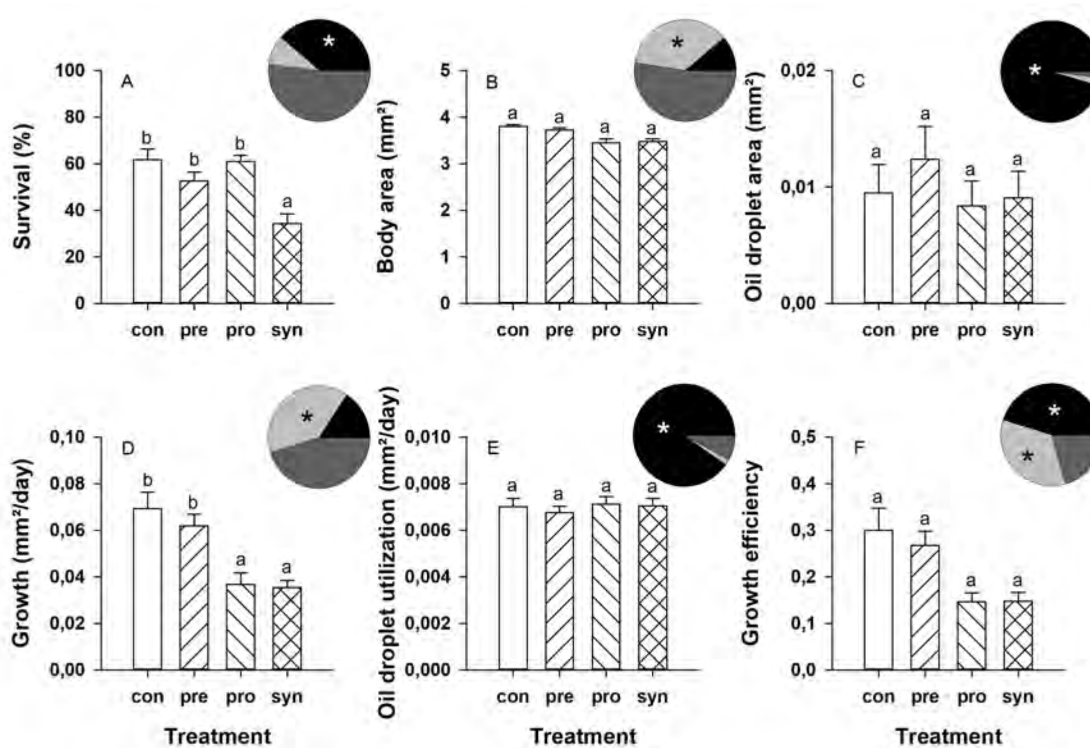
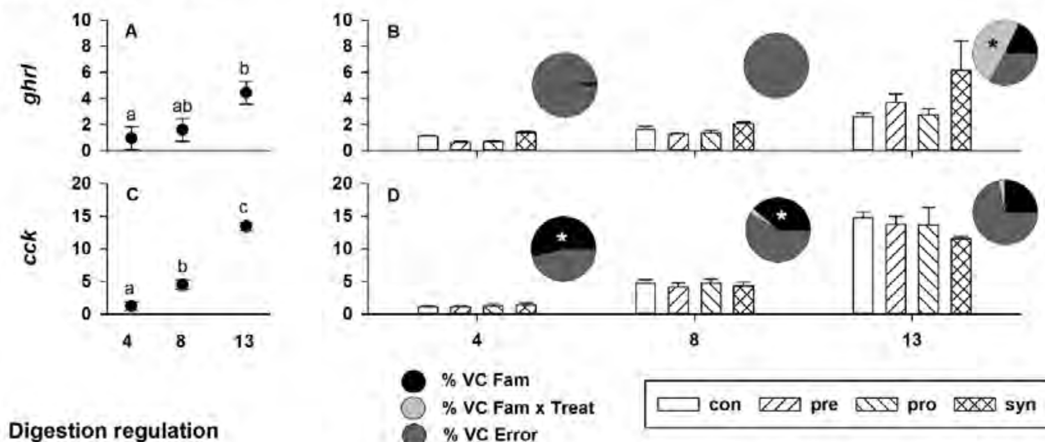


Figure 2: Survival (A), body area (B), oil droplet area (C), growth rate / day (D), oil droplet utilization / day (E), and growth efficiency (F) of European eel (*Anguilla anguilla*) larvae reared until 13 days post hatch (dph) under four gut-priming scenarios (control, pre-, pro-, and synbiotics). The main model variables were gut-priming treatment (fixed effect), family (random effect), and the treatment × family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components (VC). The VCs were generated using the Restricted Maximum Likelihood estimation method and expressed as a percentage. Black represents % VC Family, light grey represents % VC Family × Treatment and dark grey represents % VC Error. Alpha was set to 0.05.



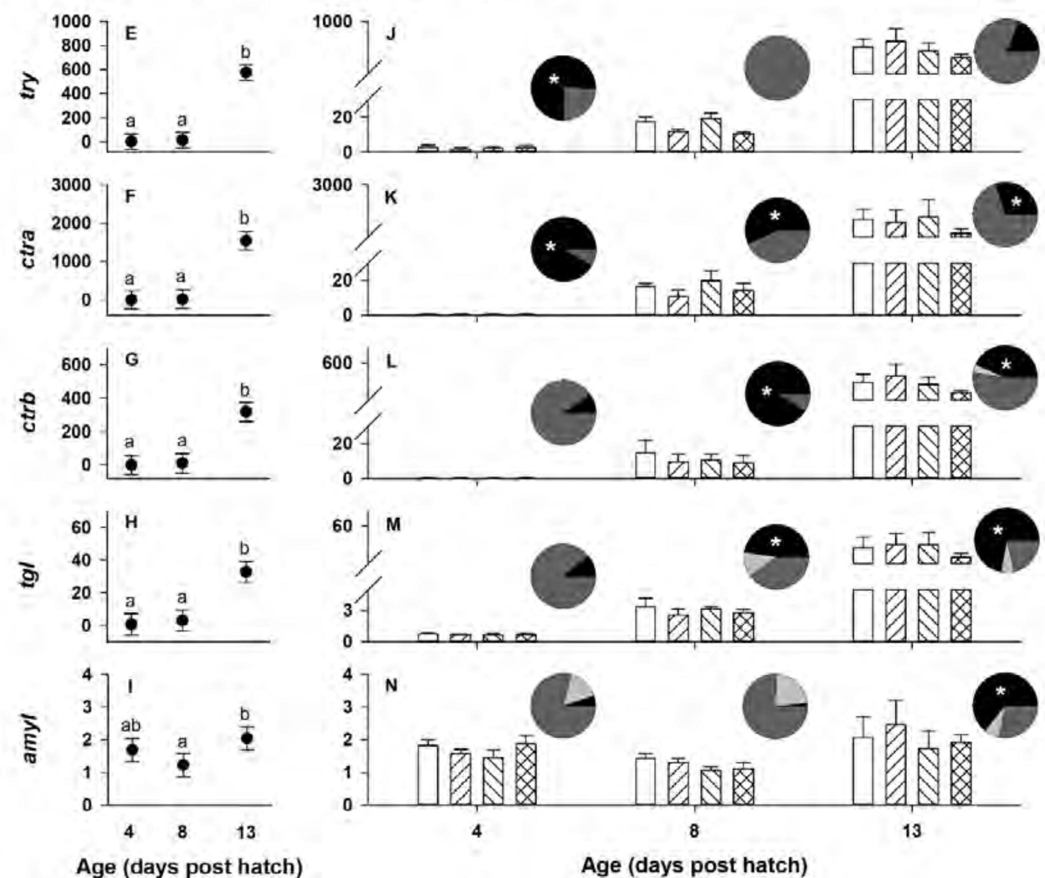


Figure 3: Expression of genes linked to appetite [ghrelin (*ghrl*)], food intake [cholecystokinin (*cck*)] and digestion [trypsin (*try*), chemotrypsin homologues (*ctra*, *ctrb*), tryglicerade lipase (*tgl*), amylase (*amyl*)] of European eel, *Anguilla anguilla* larvae at 4, 8, and 13 days post hatch reared under four gut-priming scenarios (control, pre-, pro-, and synbiotics). The main model variables were gut-priming treatment (fixed effect), family (random effect), and the treatment × family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components (VC). The VCs were generated using the Restricted Maximum Likelihood estimation method and are expressed as a percentage. Alpha was set to 0.05.

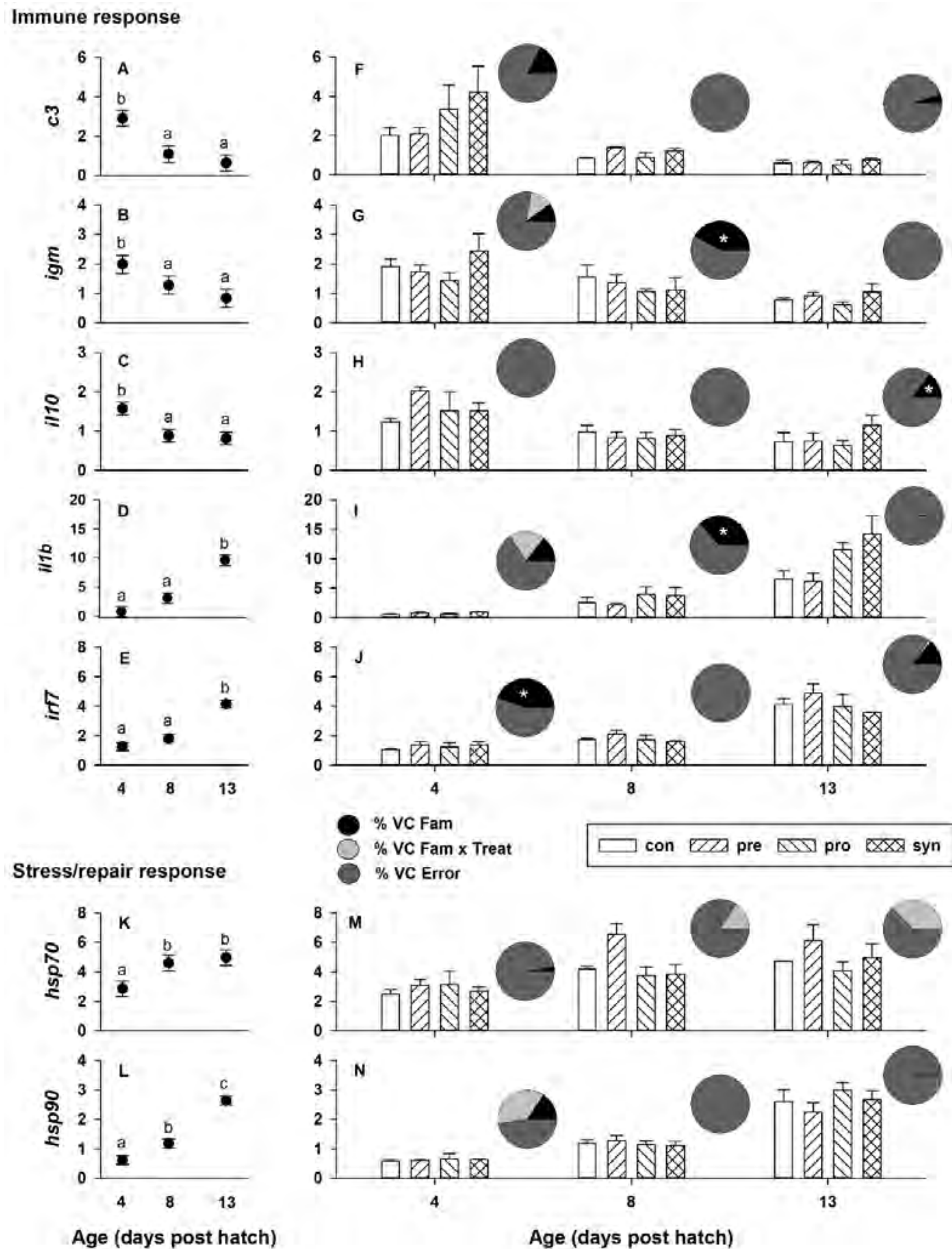


Figure 4: Expression of genes linked to stress/repair [heat shock proteins (*hsp70*, *hsp90*)] and immune response [complement component (*c3*), immunoglobulin (*igm*), interleukin 10 (*il10*), interleukin 1 β (*il1β*), interferon regulating factor 7 (*irf7*)] of European eel, *Anguilla anguilla* larvae at 4, 8, and 13 days post hatch, reared under four gut-priming scenarios (control, pre-, pro-, and synbiotics). The main model variables were gut-priming treatment (fixed effect), family (random effect), and the treatment × family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components (VC). The VCs were generated using the Restricted Maximum Likelihood estimation method and are expressed as a percentage. Alpha was set to 0.05.

Study 3:

Effects of early introduction of feeds on the transition from endogenous to exogenous feeding in European eel larvae

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Effects of early introduction of feeds on the transition from endogenous to exogenous feeding in European eel larvae

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Abstract

The transition from endogenous to exogenous feeding is a critical event during fish early life, where appropriate feed availability and timing critically influences survival. In the present study, three consecutive trials were performed, where different feeds and feeding regimes were tested for hatchery produced European eel (*Anguilla anguilla*) offspring and explored the effects of early feeding (prefeeding) during the transition from yolk sac stage to active feeding larvae. Results showed that the upregulation of *npy* and *cck* (appetite regulation) as well as the downregulation of *pomca* (food intake), observed in non-prefed larvae in Feeding regime 1 and 3, probably indicate increased likelihood of fasting and higher starvation risk. Moreover, prefeeding challenged eel larvae, resulting in reduced survival rate during endogenous feeding stage as observed in Feeding regime 2. This is further supported by the initial *hsp90* (stress/repair) upregulation in larvae receiving prefeeding during the endogenous feeding period, which was however downregulated during the exogenous feeding window, potentially revealing a capacity to adapt to emerging feeding challenges. Furthermore, the growth related *gh* expression was generally higher in prefed larvae, indicating the potential growth benefit of prefeeding. In addition, prefeeding resulted in upregulation of *pomca* (food intake) as well as *try*, *tgl*, and *amyl2a* (digestion), providing evidence of a potential benefit for eel larvae by promoting maturation of the gut functionalities. However, none of the feeding regimes seemed to include a balanced diet to result in significant larval growth, masking any initial potential benefit of the prefeeding concept. Nevertheless, larvae in Feeding regime 2, demonstrated a continuous upregulation of growth (*gh, igf1*), appetite (*cck)*, and digestion (*try*, *tgl*, *amyl*) related genes, which in combination with the highest ever registered survival (20% at 20 dph), point towards a promising dietary composition, which needs to be further developed and improved for future larviculture of European eel.

Keywords

Anguilla anguilla, prefeeding, first-feeding, growth, gene expression, hatchery technology

Highlights

- Survival rate in Feeding regime 2 was the highest ever registered in European eel larviculture
- Prefeeding challenged eel larvae, resulting in reduced survival rate during the endogenous feeding stage
- In prefed larvae *hsp90* was up-regulated during endogenous feeding, while down-regulated during exogenous feeding
- Prefeeding upregulated food intake related *pomca* as well as digestion related *try*, *tgl*, and *amyl2a*.
- Expression levels of g*h* in prefed larvae exceeded those of the control during the exogenous feeding stage

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1. Introduction

One of the challenges to be overcome to develop a sustainable aquaculture is the larval culture bottleneck. Standardized larval culture procedures have been established for a range of aquaculture species, however survival rates are often low or variable, while growth potential in most cases is not fully utilized (e.g. Conceição et al., 2010; Valente et al., 2013). Here, insufficient knowledge about nutritional requirements of first feeding fish larvae is among the key causes of high mortalities and quality issues commonly observed in larval culture (Hamre et al., 2013; Rønnestad et al., 2013; Tocher, 2010). This is particularly evident during the transition period from endogenous (utilizing exclusively yolk reserves) to exogenous feeding (exclusively relying on external feeds). During this period, fish larvae acquire the ability to feed, which involves a combination of morphological, behavioral, and physiological features (Yúfera and Darias, 2007). In addition, fish larvae start swimming and must actively search for prey, which they need to recognize and process (Kamler, 1992; Rønnestad et al., 2013). Thus, to successfully achieve the transition to exogenous feeding in culture, feeding regimes need to be tuned at the onset and timing of developmental events in the fish larvae. A delay and/or failure to establish successful first feeding may cause morphological deformities, abnormal behavior, and inability to swim or feed, leading to high mortality (Gwak and Tanaka, 2001; Kjørsvik et al., 1991; Rønnestad et al., 2013). In this regard, if feeding does not occur shortly after the exhaustion of yolk reserves, fish larvae tend to reach the point-of-no-return (PNR). Beyond this point, even if they ingest feed, the larvae may not be able to digest or assimilate and eventually perish (Kamler, 1992; Yúfera and Darias, 2007). Therefore, identifying the appropriate timing for introducing feed during larval development is pivotal to obtain robust animals at later developmental stages.

General recommendations claim that fish larvae should receive feed for the first time as soon as they achieve feeding ability, e.g. when the feeding and digestive apparatus

is developed and functional or when larvae start to swim actively in search for feed (Zambonino Infante and Cahu, 2007). Nevertheless, a delay has been observed between the time when feed was offered for the first time and tangible active ingestion by the larvae in several fish species (Garcia et al., 2020; Gisbert et al., 2004; Lima et al., 2017; Mookerji and Rao, 1999; Zhang et al., 2009). For example, Northern anchovy, *Engraulis mordax,* larvae reach the PNR only 1.5 days after yolk sac depletion and larvae are not able to survive, if feed is not introduced timely (Lasker, 1970). Similarly, hatchery-reared silver therapon, *Leiopotherapon plumbeus* have a very brief transitional feeding period (~12 h after yolk exhaustion), where initiation of feeding beyond this period results in 100% mortality (Garcia et al., 2020). Therefore, offering feed to larvae earlier than complete exhaustion of the yolk sac may be a strategy to ensure high performance of fish larvae.

In addition to the genetically preprogrammed digestive processes, food composition is also known to influence the regulation of gastrointestinal capacity during larval development (Cahu and Zambonino Infante, 2001; Rønnestad et al., 2013). For instance, in larvae of herring (*Clupea harengus*), levels of dietary protein influenced levels of cholecystokinin that control secretion of trypsin in the pancreas (Cahu et al., 2004). Similarly, in larvae of European seabass (*Dicentrarchus labrax*), the pancreatic enzymes lipase and alkaline phospholipase were stimulated by the incorporation of higher fat concentration in the diet, which promoted an early maturation of the enterocytes and consequently of the digestive tract (Vagner et al., 2009; Zambonino Infante and Cahu, 1999). In contrast, sub-optimal feeding regimes can delay the maturation of the gastro-intestinal tract and negatively affect digestive enzyme production, leading to fish larvae that are not able to cope with some dietary components due to their limited digestive capacities (Zambonino Infante and Cahu, 2007). Therefore, knowledge regarding the species-specific and ontogenetically optimal timing to introduce feeds, as well as the

nutritional predisposition and preferences is essential in any new species in aquaculture to obtain high-quality larvae.

The nutritional requirements of fish larvae differ across developmental stages, mainly due to morphological and physiological changes occurring during early ontogeny (Hamre et al., 2013; Holt, 2011). As such, the digestive capacity of fish larvae is considered limited especially in relation to the digestion of complex proteins, generally employed in fish feed (Engrola et al., 2009; Gamboa-Delgado et al., 2008; Kotzamanis et al., 2007). Thus, in order to enhance the protein assimilation by fish larvae, the use of hydrolyzed protein in feed formulations is encouraged (Cahu et al., 1999; Kolkovski and Tandler, 2000; Kotzamanis et al., 2007; Kvåle et al., 2002; Zambonino Infante et al., 1997). However, the digestive capacity of dietary protein shifts during development as observed in Senegalese sole (*Solea senegalensis*), where an increase in complexity of dietary protein is necessary to improve survival and growth rate (Canada et al., 2017). Nevertheless, inclusion of high levels of hydrolyzed protein can reduce the retention of protein and therefore decrease larval fitness (Tonheim et al., 2005) as also demonstrated for larvae of European seabass (Cahu et al., 2004), seabream, *Sparus aurata* (de Vareilles et al., 2012), Senegalese sole (Canada et al., 2017) and Atlantic halibut, *Hippoglossus hippoglossus* (Kvåle et al., 2002). Therefore, special attention should be given to the formulation of feeds in relation to the species- and stage-specific digestive capacity to identify an appropriate first-feeding regime and timing (Yúfera and Darias, 2007).

European eel (*Anguilla anguilla*) is a high-value fish species with a great potential for aquaculture, provided the establishment of hatchery technology (Tomkiewicz, 2019). Here, development of larval culture is challenged by gaps in knowledge regarding the early life history stages and their enigmatic feeding stage, i.e. the leptocephalus larvae. Therefore, the larval ontogenetic development and nutritional requirements need to be

experimentally assessed to accomplish this phase in culture (Butts et al., 2016; Lund et al., 2021; Politis et al., 2018b; Sørensen et al., 2016b). Available information about their natural diet is limited to recent analyses of gut contents of leptocephalus larvae, caught in proximity of the assumed spawning area, the Sargasso Sea (Ayala et al., 2018; Knutsen et al., 2021; Miller, 2009; Riemann et al., 2010). These studies indicate that eel larvae feed on a variety of planktonic organisms, gelatinous zooplankton, and marine snow as well as discarded appendicularian houses containing bacteria, protozoans, and other biological materials (Ayala et al., 2018). Similar challenges are posed to the closely related Japanese eel (*Anguilla japonica*), however larvae of this species have been successfully grown in culture using a diet based on shark egg yolk leading to the first production of leptocephalus larvae (Tanaka et al., 2001). Thereafter, modifications of this diet led to the first captive glass eel production (Tanaka, 2003; Kagawa et al., 2005). Since then, nutritional research for Japanese eel larvae has focused on sustainable alternatives, leading to formulation of diets based on fish protein hydrolysates (Masuda et al., 2013) or hen egg yolk and skinned Antarctic krill (Okamura et al., 2014).

For the European eel first attempts to raise larvae on an enriched rotifer paste proved unsuccessful (Butts et al., 2016), however, increased capability to produce viable larvae reaching the feeding stage has promoted progress towards first feeding culture of European eel larvae (Tomkiewicz et al., 2019). In this regard, a recent study investigating the endocrine regulation of feeding, during the transition from endogenous to exogenous feed, identified the first feeding window and described a genetically pre-programmed feeding mechanism with molecularly early maturing digestive functions (Politis et al., 2018a). Here, eel larvae expressed genes related to digestion and appetite already at 4 days post hatch (dph), indicating an adaptive potential towards a prompt maturation of the gastrointestinal function. As such, the objective of the present study was to assess the effect of early introduction of feed (prefeeding) on ingestion and digestion capacity

of European eel larvae as well as on growth and survival. Since no diet has proven effective for first feeding European eel larvae, three pioneering trials were performed, where three different formulated diets and feeding regimes were tested. Larval performance was assessed through biometry and survival as well as expression patterns of genes relating to growth [growth hormone (*gh*) and insulin-like growth factor 1 (*igf1*)], appetite [cholecystokinin (*cck*), neuropeptide Y (*npy*), ghrelin (*ghrl*)], food intake [proopiomelanocortin (*pomc*)], digestion [trypsin (*try*), triglyceride lipase (*tgl*), amylase (amyl2a)], as well as stress [heat shock protein 90 (*hsp90*)] and immune response [interleukin 1 β (*il1β*)].

1. Materials and methods

2.1 Ethic statement

All fish were handled in accordance with the European Commission's regulations concerning the protection of experimental animals (Dir 2010/63/EU). Procedures were approved by The Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696). Each fish was anesthetized before tagging, biopsy and stripping of gametes, while euthanized after stripping (females) or at the end of the experiment (males), by submergence in an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg/L, Sigma Aldrich, Germany). Larvae were anesthetized and/or euthanized using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany) at a concentration of 7.5 and 15 mg/L, respectively.

2.2 Broodstock management and offspring production

Female broodstock comprised silver eel obtained from a brackish lake, Saltbæk Vig (Zealand, Denmark), while male broodstock was raised from the glass eel stage at a commercial eel farm (Royal Danish Fish, Hanstholm, Denmark). The broodstock was transported to the EEL-HATCH facility (DTU Aqua, Hirtshals, Denmark). Upon arrival,

the fishes were acclimatized for three weeks, before assisted reproduction protocols were implemented for gamete production according to Kottmann et al. (2020a). In females, vitellogenesis was induced by weekly injections of salmon pituitary extract (Argent Chemical Laboratories, Washington, USA) at a dose of 18.75 mg/kg initial body weight, while final maturation was induced using 17a,20b-dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma-Aldrich Chemie, Steinheim, Germany) (da Silva et al., 2018; Kottmann et al., 2020b). Male broodstock received weekly injections of human chorionic gonadotropin (Sigma-Aldrich, Missouri, USA) at 1.5 IU/g of fish (Perez et al., 2000). For each female, a pool of milt from 3-5 males was obtained, sperm density was assessed and standardized using a short-term sperm storage medium (Koumpiadis et al., 2021; Peñaranda et al., 2010).

Gametes were strip-spawned and eggs fertilized at 20°C, using a standardized sperm to egg ratio with contact time of 5 min (Butts et al., 2014; Sørensen et al., 2016b). After the fertilization, eggs were transferred into 15 L containers filled with reverse osmosis water salted up to 36 psu (Aquaforest Reef Salt, Brzesko, Poland), reducing gradually the temperature to ~18°C (Sørensen et al., 2016b; Politis et al., 2018a). After 2 h, the buoyant eggs were gently transferred to 60 L black conical incubators, supplied with flow-through seawater at a flow rate of ~350 mL/min, while kept in suspension. Temperature was maintained at 18-19°C (Politis et al., 2017) and light was kept at a low intensity < 10 lux (Politis et al., 2014) throughout the incubation period. At ~48 hours post fertilization (hpf), aeration was stopped, the flow rate reduced to ~50 mL/min and larvae hatched in the incubators ~56 hpf.

Offspring abundance was estimated at 4, 24 and 48 hpf by calculating the number of eggs/embryos in 3 × 15 mL water samples collected from the incubators and extrapolated to match the total incubator volume. Estimated total numbers were used to estimate offspring production and embryonic survival (Benini et al., 2022). For

assessment of batch hatching success, subsamples of ~ 100 embryos were randomly collected from the incubators at 48 hpf. The embryos were inserted into 200 mL sterile tissue culture flasks (VWR, Denmark) filled with seawater, including rifampicin and ampicillin (each 50 mg/L, Sigma-Aldrich, Missouri, USA) to counteract microbial interference (Sørensen et al., 2014). At ~12 h after hatch, the numbers of hatched larvae versus unhatched or dead embryos were recorded and hatching success calculated as number hatched versus the total numbers in the flasks.

2.3 Selection of larval batches for the experiment

Larvae from three parental combinations were used in the experiments. Total length and weight of female eels and quality parameters for each larval batch are given in Table 1. Total length and body weight (mean ± SD) of males were 35.10 ± 3.1 cm and 90.6 ± 13.80 g, respectively (*n* = 12).

Table 1 - Female data and reproductive success, including embryonic survival at 24 and 48 hours post fertilization (hpf), hatch success (%) and amount of larvae hatched from each of the three larval batches selected for this study.

Variable	Batch 1	Batch 2	Batch 3
Female ID	6220	3627	34D1
Female weight (g)	356	778	578
Female length (cm)	59	72	69
Stripped eggs (g)	170	380	310
Eggs incubated 4 hpf (n)	222,549	476,471	80,392*
Embryonic survival 24 hpf (%)	49	71	40
Embryonic survival 48 hpf (%)	35	28	30
Hatch success (%)	88.2	65.8	83
Hatched larvae (n)	68,313	89,678	20,289

*another 75.000 eggs were transferred to a parallel experiment

2.4 Experimental rearing systems and stocking of larvae

After hatch, larvae were distributed into 77 L tanks connected to a 1.7 m^3 recirculating aquaculture system (RAS). Each RAS included a biofilter (RK Bioelements, 750 m^2, RK BioElements, Skive, Denmark), trickle filter (BioBlok 200, EXPO-NET, Hjørring, Denmark) a protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic GmbH, Bissendorf, Germany) and UV light (11 W, JBL ProCristal, Neuhofen, Germany). Temperature and salinity were maintained at 18-20°C and ~36 psu, respectively (Politis et al., 2017; 2018a) and flow rate in the tank at ~1000 mL/min, while light regime was set to constant darkness (Politis et al., 2014).

At the end of day 3 post hatch, each batch of larvae was divided into six Kreisel tanks (8 L cylindric acrylic tanks) connected to RAS (same type as described above). For each parental combination (n = 3), treatments (prefeeding vs no-prefeeding (control)) were represented by replicated Kreisel tanks (n = 3) connected to separate RAS units. Each Kreisel tank was stocked with ~800 larvae (~100 larvae/L) and the flow rates were adjusted to ~500 mL/min. The prefeeding experiment started on 4 dph. Here, half of the tanks received prefeeding, while the other half remained unfed.

At the end of day 9 post hatch, i.e. approaching the first-feeding stage, the larvae from each batch were moved to a similar set of six Kreisel tanks connected to two 0.65 m^3 RAS systems (similar to the above), but maintained at 18 psu, accommodating the feeding culture (Politis et al., 2021; Syropoulou et al., 2022). Flow rates were kept at ~500 mL/min. Temperature was maintained at 18-20°C and light (~500 lux) was only turned on during feeding (Butts et al., 2016; Okamura et al., 2019).

2.5 Prefeeding and feeding procedures

Prefeeding was performed from 4 to 9 dph. The Kreisel tanks of the prefeeding treatment received 0.05 mL of the test diet per L of rearing water, five times a day (at 2 h intervals), while the three control tanks remained unfed. The diets tested were gently pipetted into the water without stopping the water flow.

At 10 dph, corresponding to the first feeding stage at 20°C (Politis et al., 2017), larvae were fed five times a day (at 2 h intervals), where diets were gently pipetted onto the bottom of each tank, at a concentration of 0.5 mL of diet per L of rearing water. Light was gradually increased (2 min) to intensity of 21.5 ± 3.9 μmol/m^2/s (Butts et al., 2016), while water flow was paused during feeding. Larvae were allowed to feed for 30 min, after which light was dimmed off (2 min) and water flow resumed. Following a feeding period, water in the tank was set to "flow-through" for 30 min to flush away the remaining uneaten feed. Thus, new clean water, adjusted to 20°C and 18 psu, was used to refill each RAS unit. Larvae were moved into clean tanks daily.

2.6 Diet formulation and composition

All diets were freshly prepared daily. Diet A was based on pasteurized hen egg yolk (Æggeblommer, Danæg, Denmark), fish protein hydrolysate (Diana-Aqua, France) as well as a small portion of copepod and shrimp hydrolysates. Diet B was prepared using pasteurized thornback ray (*Raja clavata*) egg yolk, deskinned krill extract (Akudim, Esbjerg, Denmark) and a small portion of soybean peptides (Sgonek Biological Technology Co. Ltd, China). The krill extract consisted of defrosted and deskinned krill, mixed with reverse osmosis water at a 1:2 ratio, sieved through a nylon mesh (0.2 mm mesh size) and heat treated for 30 min at 60°C. Diet C was a mix of Diets A and B, including the same amounts of copepod and shrimp hydrolysates and similar amounts of thornback ray egg yolk, but reduced amounts of fish protein hydrolysates and krill

extract. Diet D was similar to Diet C, including the same amounts of copepod and shrimp hydrolysates as well as the same reduced amounts of fish hydrolysates and krill extract, but now replacing ray with hen egg yolk. The proximal composition of the four diets is shown in Table 2. The fatty acid composition of the diets is included in supplementary Table 1.

Table 2 - Proximal composition of the diets used in three experimental feeding regimes to prefeed and feed larvae of European eel, *Anguilla anguilla*. Ash, protein, and fat values are expressed as percentage of dry matter.

	Diet A	Diet B	Diet C	Diet D
DM (%)	34.2	15.0	31.7	22.9
Ash (% DM)	5.6	12.3	14.2	5.3
Protein (% DM)	66.0	75.2	77.4	50.0
Fat (% DM)	22.6	12.0	3.4	39.9
Energy (kJ/g DM)	26.1	20.9	20.3	28.4

2.7 Experimental design

The study includes three consecutive trials, where three feeding regimes were tested. Each trial corresponds to one parental combination. The experimental design is overviewed in Figure 1, including a time line. Each treatment, initiated on 4 dph was represented by three replicated Kreisel tanks.

Trial 1 – Feeding regime 1

The first trial applied Diet A at 0.05 mL of diet per L of rearing water during prefeeding (4-9 dph) and at 0.5 mL of diet per L of rearing water during feeding (10-20 dph). This feeding regime was formulated considering the consistent availability of stable high quality protein found in hen egg yolk as well as limited ability of fish larvae to digest native protein (Kolkovski, 2001). Thus, the inclusion of hydrolyzed proteins was hypothesized to improve survival and growth of eel larvae as demonstrated for other fish species

(Gisbert et al., 2018). The diet contained 66% of protein and 22.6% of fat as described in Table 2, while the fatty acids profile can be found in the supplementary material.

Trial 2 - Feeding regime 2

The second trial, applied Diet B at 0.05 mL of diet per L of rearing water during prefeeding (4-9 dph) and at 0.5 mL of diet per L of rearing water during feeding (10-20 dph). The use of pasteurized ray egg was inspired by the diets applied for Japanese eel larval culture (Tanaka et al., 2001). This diet contained 75% of protein and 12% of fat (Table 2).

Trial 3 - Feeding regime 3

The third trial applied a combination of Diets A, B, C and D. Diet A was used in the prefeeding treatment at 0.05 mL of diet per L of rearing water. Subsequently, both groups received Diet B from 10 to 13 dph, followed by Diet C on 14 and 15 dph and then diet D from 16 dph until the end of the trial (20 dph). This Feeding regime started with a diet (Diet A) including highly digestible protein sources (fish, copepod, and shrimp hydrolysates), followed by the differently fatty acid balanced Diet B (proven to be ingested by the larvae in Trial 2) and then switched to a transitional Diet C (a combination of Diets A and B) before applying the final Diet D, reducing the amounts of fish hydrolysates and krill extract, while replacing the ray with hen egg yolk. Diet C and D contained 77.4 and 50% of protein as well as 3.4 and 39.9% of fat, respectively (Table 2).

<u>2.8 Larval survival and biometry</u>

Dead larvae were removed daily from each replicated tank and quantified forming the basis of mortality estimates. Larval cumulative mortality was calculated based on daily mortality as a percentage from 4 dph until the end of the larval stage (20 dph).

Based on the calculation of the daily mortality, the probability of daily mortality was estimated.

Moreover, subsamples ($n = 3$) of 10 larvae each were collected from each batch ($n = 3$), anesthetized using MS-222 (Sigma-Aldrich, Missouri, USA) and photographed at hatch (0 dph), for measuring initial larval biometry. Thereafter, pools of 10 larvae per replicated tank ($n = 3$), Treatment ($n = 2$) and Feeding regime ($n = 3$) were sampled, anesthetized using MS-222 and photographed at key developmental stages; at mouth opening (8 dph), beginning of first feeding window (13 dph) and during the feeding stage (18 dph). All images were obtained using a digital camera (Digital Sight DS-Fi2, Nikon Corporation, Japan), attached to a zoom stereo microscope (SMZ1270i fitted DS-Fi2 Camera Head, Nikon Corporation, Tokyo, Japan). These images were used for morphometric measurements (body and oil drop area), using the NIS-Elements D software (Nikon Corporation, Tokyo, Japan).

2.9 Gene expression

For molecular analysis, subsamples ($n = 3$) of 10-15 larvae were collected at hatch (0 dph), from each of the batches ($n = 3$) used in this study. Moreover, 10-15 larvae from each replicated tank ($n = 3$), treatment ($n = 2$) and Feeding regime ($n = 3$) were collected at 8, 13 and 18 dph. The larvae were euthanized using MS-222, rinsed with deionized water, preserved in RNA later (Stabilization Reagent) and kept at -20°C. RNA was extracted using the NucleoSpin RNA Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. RNA concentration (264 ± 230 ng/mL) and purity (260/280 = 2.13 ± 0.03, 230/260 = 2.23 ± 0.12) were determined by spectrophotometry using NanoDrop™ One (Thermo Scientific™, USA) and normalized to a common concentration of 100 ng/mL with HPLC water. From the resulting total RNA, 400 ng were transcribed using the qScript™ cDNA Synthesis Kit (Quantabio, Germany) according to

the manufacturer's instructions, including an additional gDNA wipe out step prior to reverse transcription [PerfeCta^R DNase I Kit (Quantabio, Germany)].

The expression levels of 11 target and 2 reference genes were determined by quantitative real-time PCR (qRT-PCR). Primers were designed using primer 3 software based on cDNA sequences available in Genbank databases (Table 3). All primers were designed for an amplification size ranging from 75 to 200 nucleotides. The elongation factor 1 a (*ef1a*) and 40S ribosomal S18 (*rps18*) genes were chosen as housekeeping genes. These genes have been recommended as the most stable in fish larvae and thus, the most consistent reference genes (McCurley and Callard, 2008). Their stability was statistically confirmed, and their expression was not significantly different across treatments.

Expression of genes of all larval samples were analyzed in two technical replicates of each gene using the qPCR Biomark™ HD technology (Fluidigm) based on 48×48 dynamic arrays (GE chips). In brief, a pre-amplification step was performed with a 500 nM primer pool of all primers using the PreAmp Master Mix (Fluidigm) and 1.3 mL of cDNA per sample for 10 min at 95°C; 14 cycles: 15 sec at 95°C and 4 min at 60°C. Obtained PCR products were diluted 1:10 with low EDTA-TE buffer. The pre-amplified 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 mM. The chip was run according to the Fluidigm 48×48 PCR protocol with a Tm of 60°C. The relative quantities of target gene transcripts were normalized and measured using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Coefficient of variation (CV) of technical replicates was calculated and checked to be <0.04 (Hellemans et al., 2007).

Table 3 - Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR. Primers were designed based on sequences available on Genbank databases**.**

Full Name	*Function*	*Abbreviation*	*Primer sequence (5' 3') Forward*	*Primer sequence (5' 3') Reverse*	*Accession Number*
Elongation factor 1	Reference	*ef1*	CTGAAGCCTGGTATGGTGGT	CATGGTGCATTTCCACAGAC	XM_035428800.1
18s ribosomal RNA	Reference	*rps18*	ACGAGGTTGAGAGAGTGGTG	TCAGCCTCTCCAGATCCTCT	XM_035428274.1
Growth hormone	Growth	*gh*	GTTTGGGACCTCTGATGGGA	AGCAGGCCGTAGTTCTTCAT	XM_035398906.1
Insulin-like growth factor 1	Growth	*igf1*	TTCCTCTTAGCTGGGCTTTG	AGCACCAGAGAGAGGGTGTG	XM_035427391.1
Cholecystokinin	Appetite	*cck*	CGCCAACCACAGAATAAAGG	ATTCGTATTCCTCGGCACTG	XM_035409023.1
Prepro-Ghrelin	Appetite	*ghrl*	TCACCATGACTGAGGAGCTG	TGGGACGCAGGGTTTTATGA	XM_035381207.1
Proopiomelanocortin v1 and v2	Food intake	*pomca*	GCCTGTGCAAGTCTGAACTG	GACACCATAGGGAGCAGGAA	XM_035421304.1
Neuropeptide Y	Appetite	*npy*	CCGCATTGAGACACTACATCA	GGTGAGACGGCAAAACTGAA	XM_035429113.1
Amylase	Digestion	*amyl2a*	AGACCAACAGCGGTGAAATC	TGCACGTTCAAGTCCAAGAG	XM_035420193.1 v3
Triglyceride lipase	Digestion	*tgl*	CTGACTGGGACAATGAGCGT	CGTCTCGGTGTCGATGTAGG	XM_035399731.1
Trypsin	Digestion	*try*	CTGCTACAAATCCCGTGTGG	GGAGTTGTATTTGGGGTGGC	XM_035429595.1
heat shock protein 90	Stress/Repair	*hsp90*	ACCATTGCCAAGTCAGGAAC	ACTGCTCATCGTCATTGTGC	XM_035392491.1 v2
Interleukin 1β	Immune response	*il1β*	ATTGGCTGGACTTGTGTTCC	CATGTGCATTAAAGCTGACCTG	XM_035380403.1 v2

2.10 Statistical analyses

All data were analyzed using R studio statistical analysis software (Version 1.3.959, *RStudio: Integrated Development for R. RStudio, PBC, Boston, MA*). Residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted values) to ensure they met model assumptions. Data were log(10) transformed to meet these assumptions when necessary. Alpha was set at 0.05 for testing main effects and interactions. Treatment means were contrasted using Tukey's Honest Significant Difference test. Body area and oil droplet area as well as gene expression

(15 genes) were analyzed using a series of mixed model ANOVAs (PROC GLM)(Brooks et al., 2017). The main model variables (treatment (control vs. prefeeding)) and age (0, 8, 13, 18 dph) were analyzed for every Feeding regime (1, 2 and 3), while replicated tanks were considered random. The initial model tested, included an interaction effect between treatment and age. The model was reduced when possible. The final model was validated through analyses of the residuals.

For survival, t-tests were run at key developmental stages; at 9 dph to evaluate the solemnly treatment effect of prefeeding, and at 20 dph to evaluate the combined effect of prefeeding and feeding. Moreover, the model for survival was set up as a sequential binomial model, where the X_{ij} as the amount of dead larvae in the *i´*th experimental tanks where *i*= 1…. 18 at the *j´*th age where *j*= 4…20 dph. The observations are described by

$$X_{ij} \sim \text{Bin}(N_{ij}, P_{ij})$$

where

$$\text{logit}(P_{ij}) = \alpha(\text{Diet}_i, \text{Treatment}_i) * \Delta t_j \quad j = 4 \ldots 20\text{dph}$$

Here, N_{ij} is the number of survival in experimental tank i at the time j and the Δt_j is the length of the time interval from the *j*-1 to the *j´*th age. The final model used for this analysis includes an interaction effect (treatment × diet) that could not be further reduced.

3. Results

3.1 Survival

The survival of larvae (presented as mean ± SEM of the three replicated tanks) per Feeding regime, from 4 to 20 dph, is illustrated in Figure 2. Here, during the prefeeding period (from 4 to 9 dph), larval survival for control treatments was similar among batches, with mean values on 9 dph of 57.24 ± 9.38 %, 60.38 ± 1.29 %, and 58.6 ± 3.09 % for Feeding regimes 1 – 3, respectively. Comparatively, survival in the prefeeding treatments was 40.48 ± 10.47 %, 50.54 ± 2.28 % and 57.99 ± 0.43 % for Feeding regimes 1-3, respectively. The differences between the control and prefeeding treatments in feeding regimes 1 and 3 were not significant, neither at the end of the

endogenous (9 dph) nor within the exogenous feeding stage (Fig. 2A and 2C). However, in Feeding regime 2 (Fig. 2B), the survival of larvae at the end of the endogenous feeding stage (9 dph) was significantly higher in the control group compared to the prefeeding group ($p < 0.01$), however the effect diminished during the exogenous feeding phase and was not longer significant at 20 dph ($p = 0.276$). Interestingly though, the mortality probability was significantly increased ($p < 0.01$), when prefeeding was applied in the feeding regimes 1 and 2, while this was not detectable in Feeding regime 3 (Table 4).

Table 4 - European eel, Anguilla anguilla larval mortality probability in relation to prefeeding treatment in three different feeding regimes calculated from 4 to 20 dph (± SEM) and associated p-value.

	Ctrl	Pre	*P-value*
Feeding regime 1	0.102 ± 0.0040	0.112± 0.004	< 0.01*
Feeding regime 2	0.167 ± 0.0055	0.200± 0.006	< 0.01*
Feeding regime 3	0.121 ± 0.0055	0.122 ± 0.004	0.92

3.2 Biometry

Larval biometrics (body and oil droplet area) in three different feeding regimes are shown in Figure 3. Here, larval body area significantly ($p < 0.00001$) increased within the endogenous feeding stage in all feeding regimes (Fig. 3 A, F, K). Thereafter, in feeding regimes 2 and 3, larvae showed a lower but continuous growth rate within the exogenous feeding period (Fig. 3 F and K), while in Feeding regime 1, larval body area did not change beyond 8 dph (Fig. 3 A). At the same time, in Feeding regime 2, body area of non-prefed larvae (control group) was significantly larger ($p < 0.01$) than for prefed larvae (Fig. 3 G). This phenomenon was not detected in larvae from Feeding regime 1 or 3 (Fig. 3 B and I). Concurrently, the oil droplet area significantly ($p < 0.01$) decreased throughout the endogenous feeding stage (Fig. 3 C, H, L), but was not affected by the treatment in any of the Feeding regimes (Fig. 3 D, J, M).

3.3 Stress/repair, immune and growth-related gene expression

The stress/repair, immune and growth-related gene expression in the three feeding regimes is illustrated in Figure 4.

In Feeding regime 1, *hsp90* and *il1β* expression was not affected by treatment (Fig. 4B and D), but significantly ($p < 0.01$) increased from hatch and peaked on 18 dph (Fig. 4A and C). In Feeding regime 2, *hsp90* and *il1β* expression was also not affected by treatment (Fig. 4F and H), while significantly ($p < 0.01$) increased from hatch to 8 dph but remained stable the remaining ontogenetic period investigated (Fig. 4E and G) at a several-fold lower level than in Feeding regime 1. In Feeding regime 3, a significant ($p < 0.01$) age × treatment interaction was detected for the expression pattern of *hsp90*. Therefore, the effect of age at each treatment is illustrated in Figures 4I and J, while the effect of treatment at each age is illustrated in Figure 4K. Here, *hsp90* expression remained low in non-prefed larvae and only significantly ($p < 0.01$) increased on 18 dph (Fig. 4I), while for prefed larvae *hsp90* expression was significantly ($p < 0.01$) upregulated on 8 and 18 dph (Fig. 4J). Consequently, in prefed larvae, *hsp90* expression was significantly ($p < 0.01$) higher on 8 dph, but significantly ($p < 0.01$) lower on 13 and 18 dph compared to non-prefed larvae (Fig. 4K). At the same time, *il1β* expression, which was not affected by treatment (Fig. 4M), significantly ($p < 0.01$) increased from hatch to 8 dph and remained stable until 18 dph (Fig. 4 L). Markedly, the expression of *hsp90* was approximately 3-fold, while the expression of *il1β* was approximately 5-fold higher in Feeding regime 1 compared to the other Feeding regimes.

Regarding *gh* expression, a significant ($p < 0.01$) age × treatment interaction was detected in Feeding regime 1. Therefore, the effect of age at each treatment is illustrated in Figures 4N and O, while the effect of treatment at each age is illustrated in Figure 4P. In non-prefed larvae (control), expression of *gh* was significantly ($p < 0.01$) upregulated on 13 dph and remained stable until 18 dph (Fig. 4N), while for prefed larvae it was

significantly ($p < 0.01$) upregulated on 13 dph and decreased at 18 dph (Fig. 4O). Consequently, in prefed larvae, *gh* expression was significantly ($p < 0.01$) higher on 13 dph, but significantly ($p < 0.01$) lower on 18 dph compared to non-prefed larvae (Fig. 4P). In Feeding regime 2, *gh* expression was significantly ($p < 0.01$) upregulated in prefed larvae (Fig. 4T) and significantly ($p < 0.01$) and continuously increased approximately 20000-fold throughout ontogeny, reaching peak values on 18 dph (Fig. 4S). In Feeding regime 3, a significant ($p < 0.01$) age × treatment interaction was detected. Therefore, the effect of age at each treatment is illustrated in Figures 4W and X, while the effect of treatment at each age is illustrated in Figure 4Y. Here, expression of *gh*, was significantly ($p < 0.01$) upregulated beyond 13 dph for both, non-prefed and prefed larvae (Fig. 4W and X). Consequently, *gh* expression was significantly ($p < 0.01$) higher in prefed compared to non-prefed larvae on 18 dph (Fig. 4Y). Markedly, the expression of *gh* was approximately 2-fold higher in Feeding regime 2 compared to the other Feeding regimes. On the other hand, *igf1* expression was not affected by treatment (Fig. 4R, V, AA), but significantly ($p < 0.01$) upregulated on 8 dph in all feeding regimes (Fig. 4 Q, U, Z). Interestingly, *igf1* expression significantly ($p < 0.01$) decreased again beyond that point in Feeding regimes 1 and 3 but remained 2 to 3-fold higher upregulated in Feeding regime 2.

3.5 Appetite and food intake related gene expression

The expression of genes related to appetite and food intake in the three feeding regimes are illustrated in Figure 5.

In Feeding regime 1, a significant ($p < 0.01$) age × treatment interaction was detected for the expression pattern of *cck*. Therefore, the effect of age at each treatment is illustrated in Figures 5A and B, while the effect of treatment at each age is illustrated in Figure 5C. Here, *cck* expression significantly ($p < 0.01$) increased on 8 dph and remained

stably upregulated at this level until 18 dph for non-prefed larvae (Fig. 5A), while *cck* expression significantly ($p < 0.01$) increased throughout development and peaked at 13 dph but was downregulated again at 18 dph for prefed larvae (Fig. 5B). Consequently, *cck* expression was significantly ($p < 0.01$) lower in prefed compared to non-prefed (control) larvae on 18 dph (Fig. 5C). On the other hand, *cck* expression was not affected by treatments (Fig. 5L and T), but significantly ($p < 0.01$) increased continuously until 13 dph (Fig. 5I and S) in Feeding regimes 2 or 3. Interestingly, beyond that point, *cck* expression remained stable until 18 dph in Feeding regime 3 (Fig. 5S), while it continued to rise in Feeding regime 2 (Fig. 5I), reaching an almost 2-fold higher expression compared to the other Feeding regimes.

Moreover, in Feeding regime 1, a significant ($p < 0.01$) age × treatment interaction was detected for *npy* expression. Therefore, the effect of age at each treatment is illustrated in Figures 5D and E, while the effect of treatment at each age is illustrated in Figure 5F. Here, *npy* expression significantly ($p < 0.01$) decreased throughout ontogeny for both, non-prefed and prefed larvae (Fig. 5D and E), while it was significantly ($p < 0.01$) lower in prefed compared to non-prefed (control) larvae on 18 dph (Fig. 5F). On the other hand, *npy* expression was unaffected by treatment (Fig. 5N) and significantly ($p < 0.01$) decreased throughout ontogeny, reaching basal levels beyond 13 dph in Feeding regime 2 (Fig. 5M). In Feeding regime 3, the expression of *npy* followed a similar significant ($p < 0.01$) decreasing fashion (Fig. 5U), while prefed larvae showed a significant ($p < 0.01$) continuous downregulation compared to non-prefed larvae (Fig. 5V). At the same time, *ghrl* expression generally followed a significant increasing trend throughout ontogeny (Fig. 5G, O, W), but was unaffected by treatments (Fig. 5H, P, Y) in all Feeding regimes.

Furthermore, *pomca* expression was significantly ($p < 0.01$) upregulated in prefed compared to non-prefed larvae in Feeding regimes 1 and 3 (Fig.5K and Z) but was

unaffected by treatment in Feeding regime 2 (Fig. 5P). Regarding the expression pattern, in Feeding regime 1, *pomcα* significantly ($p < 0.01$) increased on 8 dph, reaching constant levels until 18 dph (Fig. 5J), while in Feeding regime 3, which followed a similar trend until 13 dph, a significant ($p < 0.01$) upregulation was observed at 18 dph (Fig. 5X). Interestingly though, *pomcα* expression significantly ($p < 0.01$) and continuously increased approximately 90-fold throughout ontogeny in Feeding regime 2, reaching peak values on 18 dph (Fig. 5Q), which are approximately 2-fold higher than in Feeding regime 3 and approximately 3-fold higher than in Feeding regime 1.

3.6 Digestion related gene expression

The expression of genes encoding the major digestive enzymes in the three feeding regimes are illustrated in Figure 6.

In Feeding regime 1, a significant ($p < 0.01$) age × treatment interaction was detected for the expression patterns of *amyl2a* and *try*. Therefore, the effect of age at each treatment is illustrated in Fig. 6A, B and Fig. 6F, G, respectively, while the effect of treatment at each age is illustrated in Fig. 6C and Fig.6H, respectively. Here, *amyl2a* and *try* expression levels significantly ($p < 0.01$) increased on 13 dph and remained stably upregulated at this level until 18 dph for non-prefed larvae (Fig. 6A, F), while *amyl2a* and *try* expression levels significantly ($p < 0.01$) increased at 13 dph but were downregulated again at 18 dph for prefed larvae (Fig. 6B, G). Moreover, *amyl2a* and *try* expression levels were significantly ($p < 0.01$) higher in prefed compared to non-prefed (control) larvae on 13 dph (Fig. 6C, H). On the other hand, *tgl* expression was not affected by treatments (Fig. 6E), while showing a significant ($p < 0.01$) 12-fold increase throughout the exogenous feeding period (Fig. 6E).

In Feeding regime 2, expression of all digestion related genes (*amyl*, *tgl*, and *try*) significantly ($p < 0.01$) increased several-fold throughout ontogeny, especially within the

exogenous feeding window and peaked at 18 dph (Fig. 6J, I and M), reaching values that were approximately 2-fold higher than in the other Feeding regimes. Moreover, *amyl* and *try* expression was not affected by treatment (Fig. 6K and N), but *tgl* expression was significantly ($p < 0.01$) upregulated in prefed compared to non-prefed (control) larvae (Fig. 6L).

In Feeding regime 3, a significant ($p < 0.01$) age × treatment interaction was detected for the expression patterns of all investigated digestion related genes (*amyl*, *tgl*, and *try*). Thus, the effect of age at each treatment is illustrated in Fig. 6P, Q, S, T, V and W, while the effect of treatment at each age is illustrated in Fig. 6R, U and X. Here, irrespective of treatment, expression of all digestion related genes (*amyl*, *tgl*, and *try*) was significantly ($p < 0.01$) upregulated on 13 dph but remained stable throughout the exogenous feeding period in non-prefed (control) larvae (Fig. 6P, S, V). On the contrary, expression of all digestion related genes (*amyl*, *tgl*, and *try*) was significantly ($p < 0.01$) upregulated further at 18 dph in prefed larvae (fig. 6Q, T, W). Consequently, *tgl* and *try* expression was significantly ($p < 0.01$) higher in non-prefed larvae on 13 dph (Fig. 6 U, X), but expression of all digestion related genes (*amyl*, *tgl*, and *try*) was significantly ($p < 0.01$) higher in prefed larvae on 18 dph (Fig. 6R, U, X)

4. Discussion

This study experimentally tested different feeds and Feeding regimes for European eel larval culture and explored the effects of early feeding (prefeeding) during the transition from endogenous to exogenous feeding stage. In this regard, establishing a first feeding culture of European eel is at a pioneering state, where successful production of larvae of uniform quality, has only recently enabled feeding experiments (Tomkiewicz et al., 2019).

4.1 Survival

In the present study, results show that the early introduction of feeding can affect larval survival rate, as observed in Feeding regime 2. Here, during the endogenous feeding stage, larvae receiving prefeeding had a lower survival compared to the control. However, no difference in survival was noticed at the end of the experiment. High mortality rates during the larval stage are commonly registered in nature as well as in aquaculture, despite the absence of predators, the environmental stability and the constant food availability providing better survival conditions (Peck et al., 2015). This is, to a large extend, connected to morphological and physiological changes, and often related to successful or unsuccessful molecular responses in the quest to adapt to new challenges (McMenamin and Parichy, 2013). In this regard, the early introduction of feed might challenge the larvae during a very delicate moment of their life, when the immune response as well as gut functionality are still under development. As such, while for some larvae getting in contact with feed (and the associated microflora) for the first time does not necessarily provoke any adverse physiological changes, for some other, more "fragile" larvae, prefeeding can be more challenging and possibly lead to fatality.

4.2 Stress/repair response

The expression of *hsp90* is considered a reliable indicator of the stress/repair mechanism activated by external stressors, as also described by Cara (et al., 2007). Results from this study showed that after the initial *hsp90* upregulation, driven by the early introduction of feed, prefed larvae showed a repairing capacity demonstrated by the downregulation at 13 and 18 dph compared to non-prefed larvae (Feeding regime 3). However, stress during early life history can compromise development, as stressed larvae spend energy to restore and/or maintain homeostasis, which is then not further available to be invested into growth (Guderley and Pörtner, 2010). As such, in the current

study, the potential stress caused by early introduction of feed might have affected eel larval growth as observed for larvae receiving prefeeding in Feeding regime 2. This is in contrast to studies on silver catfish, *Rhamdia voulezi* (Lima et al., 2017), obscure puffer, *Takifugu obscurus* (Shi et al., 2010) and Senegalese sole, *Solea senegalensis* (Engrola et al., 2009) larvae, where earlier feed introduction resulted in improved growth. Therefore, larvae offered prefeeding may show higher adaptability to new challenges, possibly by preparing the larvae to accept a full-scale feeding regime, but suboptimal procedures might cause high levels of stress during the transitional period between endogenous and exogenous feeding, leading to impaired growth.

4.3 Growth

In the present study, growth related *gh* expression was upregulated in prefed larvae right after the prefeeding stage (on 13 dph) in Feeding regime 1, towards the end of the first feeding window (on 18 dph) in Feeding regime 3 and during the entire period in Feeding regime 2. Moreover, *igf1* expression, which was approximately 100-fold upregulated on 8 dph in all feeding regimes, decreased during the exogenous feeding period in Feeding regimes 1 and 3, but remained 2 to 3-fold higher upregulated in Feeding regime 2. Thus, the prefeeding principal, especially in Feeding regime 2 seems to have stimulated a molecular signal for growth potential, which, however, was not translated into morphological growth performance.

4.4 Appetite and food intake

Larval growth also relies on mechanisms and processes relating to appetite and feed intake (Kestemont and Baras, 2001). In the present study, appetite related *ghrl* was not affected by prefeeding, but in non-prefed larvae *npy* was upregulated throughout the entire period in Feeding regime 3, while *npy* and *cck* were upregulated towards the end

of the first feeding window (on 18 dph) in Feeding regime 1. In this regard, the expression of *npy*, which acts as an appetite stimulator (orexigenic factor), is commonly associate to fasting (Assan et al., 2021), while *cck*, which acts as an appetite inhibitor (anorexigenic factor), tends to be downregulated when the gut is full and upregulated when it is empty (Tillner et al., 2013). On the contrary, feed intake related *pomca*, which was proven to be a good biomarker to demonstrate feed intake in eel larvae (Politis et al., 2018b), was in the present study downregulated throughout the entire period for larvae not receiving prefeeding in Feeding regimes 1 and 3. Consequently, the higher expression of *npy* and *cck* as well as the lower expression of *pomca* observed in non-prefed larvae in the present study, probably indicate a likelihood of fasting and higher starvation risk. As such, we here provide evidence that the introduction of feed before mouth opening can influence appetite and feed intake related mechanisms in eel larvae, promoting the importance of the prefeeding principle.

4.5 Digestion

The transcription of genes encoding the major digestive pancreatic enzymes, such as trypsin, lipase, and amylase (overviewed in Figure 7A, B, C), increased from basal levels during the endogenous feeding period to peak levels during the exogenous feeding period, confirming the molecular transition into the first-feeding window. This is a process, which is typically connected to genetically pre-programmed mechanisms related to digestion (Politis et al., 2018b), but can also be influenced by dietary composition (Zambonino Infante and Cahu, 2007). In the present study, prefed larvae showed upregulated *tgl* expression throughout the entire period in Feeding regime 2, upregulated *try* and *amyl2a* expression at 13 dph in Feeding regime 1, as well as upregulated expression of all three digestion related genes (*try*, *tgl*, *amyl2a*) at 18 dph in Feeding regime 3. Therefore, the earlier introduction of feed increased the production of

digestive enzymes, thus, probably supported the maturation of the gastro-intestinal tract and prepared larvae to digest and assimilate nutrients. Furthermore, the expression levels of *try* were generally much higher compared to *tgl* and *amyl2a*, confirming the eel larval nutritional necessity for protein during this life stage, as previously described for European and Japanese eels (Hsu et al., 2015; Politis et al., 2018b).

4.6 General consideration about the diets and feeding regimes

Overall, we could recognize some general patterns of gene expression as shown in Figure 7. As such, in Feeding regime 1, the standardized expression of genes relating to growth (*gh, igf1*), appetite (*cck)*, and digestion (*try*, *amyl*) showed a similar pattern (Fig. 7A), where transcription unexpectedly "dropped" beyond 13 dph in prefed larvae. Here, the diet used in Feeding regime 1, which was based on hen egg yolk, seemed to be less attractive for European eel larvae to successfully initiate feeding and thrive throughout the first-feeding window. Moreover, the standardized expression of *il1β* and h*sp90* (Fig. 7D, E and F), was 4-fold higher in Feeding regime 1 compared to the others, revealing an immune and stress/repair response, potentially indicating the unsuitability of this Feeding regime. On the contrary, larvae in Feeding regime 2 showed a continuous upregulation of growth (*gh, igf1*), appetite (*cck)*, and digestion (*try*, *tgl*, *amyl*) related genes, which in combination with the highest recorded survival values, indicate a positive effect of this Feeding regime. Here, Diet B used in this Feeding regime had a much higher inclusion of protein (75%) and a much lower amount of lipid (12%) compared to Diet A and D (used in Feeding regimes 1 and 3), where it was 66 and 50% for protein and 22.6 and 40% for lipid, respectively. Thus, the composition of Diet B seems to be more adapted towards eel larval requirements, but the feed formulation and dietary regime still need to be further developed and improved for future larviculture of European eel.

Moreover, in Feeding regime 3, larvae were "tricked" to successfully initiate first feeding by applying Diet B, the palatability of which was proven in Trial 2, followed by the transitional Diet C (a combination of Diets A and B), before applying the final and more balanced (50% protein and 40% lipid) Diet D, where fish hydrolysates were reduced and ray egg yolk replaced with hen egg yolk. However, we did not observe any benefit for larvae in this Feeding regime, probably due lack of attraction towards the hen egg yolk and/or partly inappropriate nutritional value necessary to sustain larval growth and survival, leading to unavoidable mortality beyond the point-of-no-return. In this regard, hydrolyzed proteins, have been shown to promote the development of the digestive and immune system (Gisbert et al., 2018), but too high dietary levels of hydrolysates can produce an overload of amino acids and peptides in the intestine, which could induce the saturation of peptide transporter mechanisms (Cahu et al., 1999; Canada et al., 2019). As such, inappropriate inclusion levels of such proteins can also have negative effects on growth and survival of fish larvae, as shown for gilthead sea bream, *Sparus aurata* (de Vareilles et al., 2012) and Nile tilapia, *Oreochromis niloticus* (da Silva et al., 2017). Therefore, an early introduction of protein hydrolysates in the diet could potentially improve the digestive capacity of eel larvae at early stages, but at later developmental stages, the high concentration of small dietary peptides does not necessarily seem to improve survival and growth.

4.7 Microbial management

Keeping high water quality is pivotal for eel larviculture (Sørensen et al., 2014; Sørensen et al., 2016). However, high animal densities in culture, in combination with feeding procedures tend to facilitate the opposite, potentially causing growth of opportunistic bacteria, which can induce an infection and/or affect the development of the microbial larval gut community (Olafsen, 2001). Here, the dietary composition can

also influence the microflora composition, resulting in the formation of a specific local microflora and/or proliferation of bacteria that normally have restricted opportunities to grow (Rowland et al., 2018). As such, the early introduction of feed can involve an early microbial interplay between host, feed, and water, when the fish larval immune system is still forming (Vadstein et al., 2018). On the contrary, the natural habitat of eel larvae (the Sargasso Sea) is oligotrophic, with low levels of bacteria and nutrients. Thus, despite the major development of aquaculture systems in removing nutrients from the rearing water and providing a balanced microbial community, captive reared eel larvae will always be challenged by high stocking densities (~100 larvae/L) and load of nutrients, in connection to increased leaching from liquid diets, resulting in lower fitness compared to nature.

5. Conclusions

To summarize, the initial stress/repair related *hsp90* upregulation, observed in prefed larvae during the endogenous feeding period, followed a downregulation during the exogenous feeding window, probably revealing adaptability to new challenges, due to being "primed" when introduced to the feeding regime. This is further supported by the early introduction of feed (prefeeding) resulting in downregulation of appetite related *npy* and *cck,* but also upregulation of food intake related *pomca* as well as digestion related *try*, *tgl*, and *amyl2a*. Therefore, the prefeeding concept supported the maturation of the gastro-intestinal tract functionality and equipped early larvae with an improved digestive capacity. At the same time, prefeeding triggered a molecular signal for growth potential, based on the upregulation of *gh*, but none of the feeding regimes seemed to provide a balanced diet to result in biometrical larval growth, masking any potential initial benefit of prefeeding. In this regard, diets based on high levels of hydrolyzed proteins might not be suitable to promote assimilation into growth and/or sustain survival during later stages

of development. However, while the Feeding regime 1 and 3 seemed to be unsuitable for first-feeding eel larviculture, larvae fed with Diet B in Feeding regime 2, demonstrated a continuous upregulation of growth (*gh, igf1*), appetite (*cck)*, and digestion (*try*, *tgl*, *amyl*) related genes, reaching values several-fold higher than in the other Feeding regimes. Moreover, this was the first time that a 20% survival was registered at 20 dph, pointing towards a dietary composition, getting closer to the nutritional requirements of first-feeding European eel larvae. Nevertheless, the feed formulation and dietary regime need to be further developed and improved for future larviculture of this species.

In conclusion, even if the Feeding regimes tested were not optimally adapted for eel larvae, the present study provided information on the potential benefit of prefeeding, by promoting an early maturation of the gut functionality, while generally improved the knowledge regarding eel larval nutritional requirements, from a morphological and molecular point of view. However, more research efforts are needed to find a suitable first-feeding diet for European eel larvae and further investigate dietary options and application timing of prefeeding as well as improve the rearing methods to ensure highest water and offspring quality possible.

CRediT authorship contribution statement

All authors contributed to this manuscript. Elisa Benini: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. Sofia Engrola: Conceptualization, Methodology, Data curation, Validation, Writing – review & editing, Supervision. Sebastian Nikitas Politis: Conceptualization, Methodology, Investigation, Data curation, Validation, Writing – review & editing, Supervision, Funding acquisition. Sune Riis Sørensen: Methodology, Resources, Investigation, Visualization, Writing – review & editing, Funding acquisition. Anders Nielsen: Formal analysis, Data curation, Validation, Writing – review & editing. Luis E.C. Conceição: Conceptualization, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing. André Santos: Data curation, Validation, Writing – review & editing. Jonna Tomkiewicz: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests.

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FIGURE LEGENDS

<table>
<tr><th></th><th></th><th colspan="2">Feeding regime 1</th><th colspan="2">Feeding regime 2</th><th colspan="2">Feeding regime 3</th></tr>
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Figure 1. Schematic overview of three European eel (*Anguilla anguilla*) larval feeding regimes (1-3) and treatments applied from 0 to 20 days post hatch (dph).

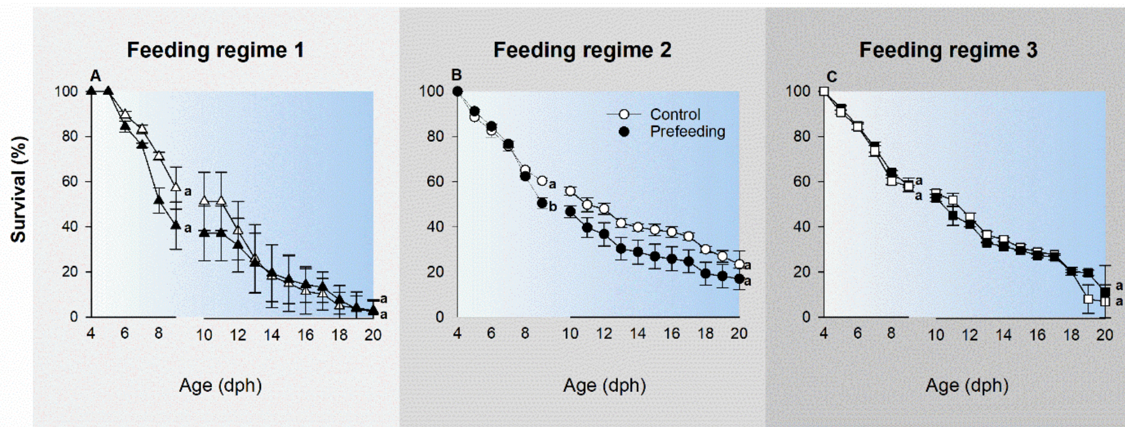

Figure 2. European eel, *Anguilla anguilla* larval survival for three feeding regimes (1, 2 and 3) and two treatments (control vs prefeeding) from 0 to 20 days post hatch (dph). Different shades of grey represent the different dietary regimes. The transition between endogenous and exogenous feeding stage is indicated by shades of blue. Values represent means (± SEM) of survival percentage, while values with different letters are significantly different at alpha = 0.05.

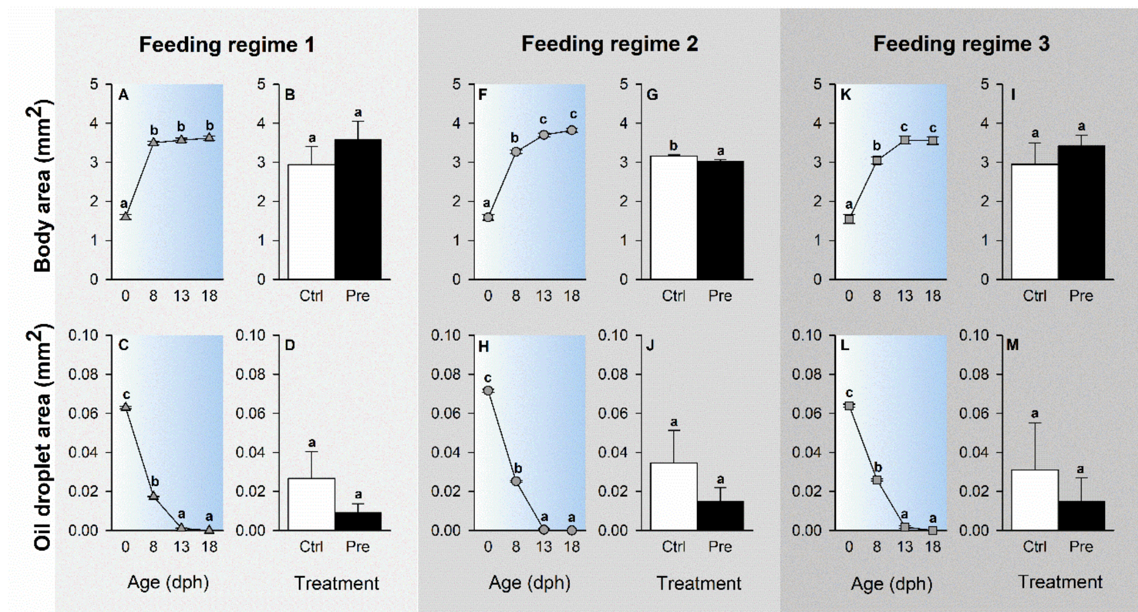

Figure 3. Effect of age in days post hatch (dph) and treatment (prefeeding (Pre) vs control (Ctrl)) on European eel, *Anguilla anguilla* larval biometrics (body and oil droplet area) in three different feeding regimes. Shades of blue indicate the transitional period from endogenous to exogenous feeding. Values represent means (± SEM) of body area and oil droplet area, while different letters represent significant differences at alpha = 0.05.

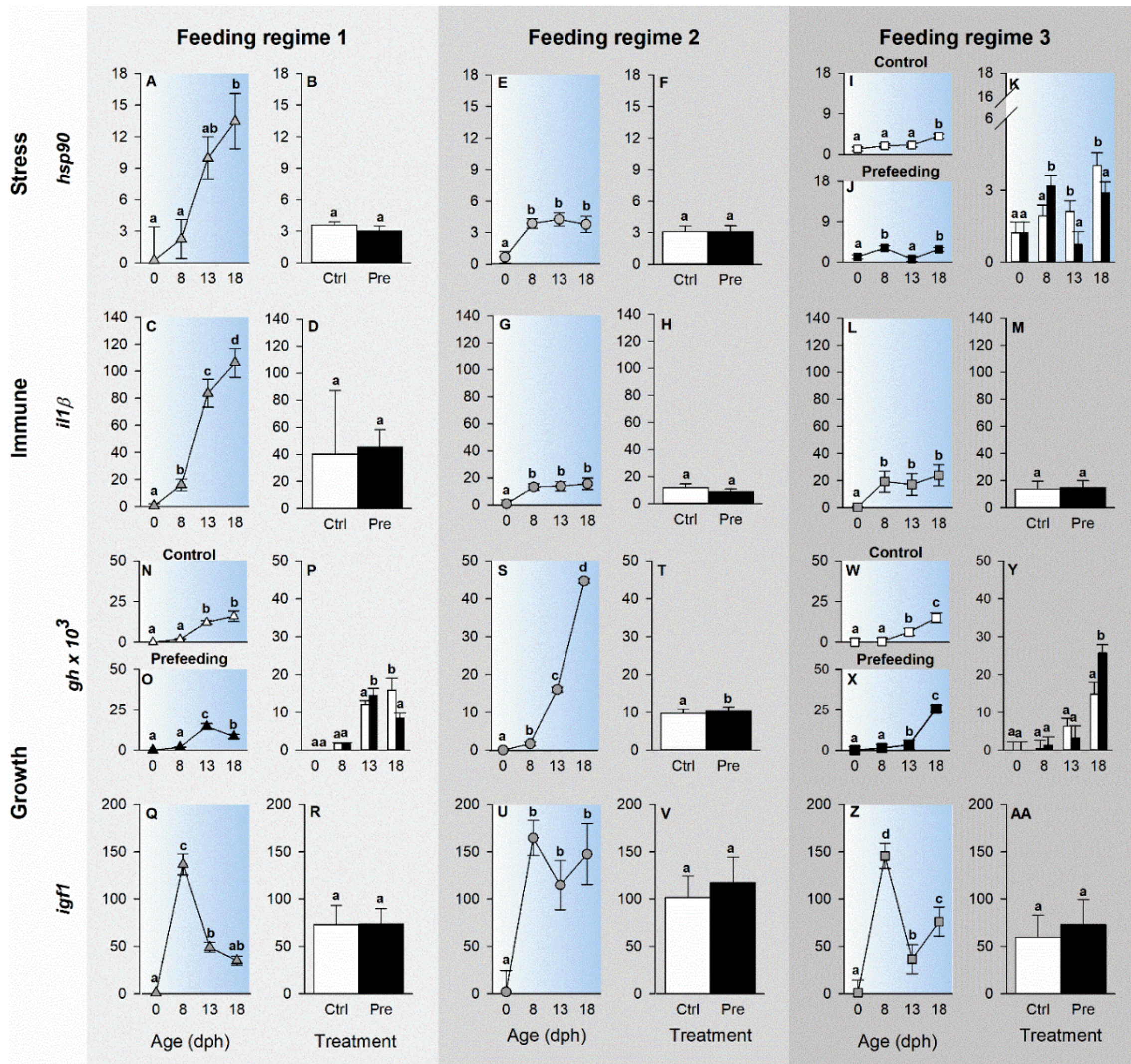

Figure 4. Effect of age in days post hatch (dph) and prefeeding treatment (control (Ctrl) vs. prefeeding (Pre)) on European eel, Anguilla anguilla larval stress, immune and growth-related gene expression in three different feeding regimes (1, 2 and 3). Relative expression of heat shock protein 90 (hsp90), interleukin 1 beta (il1b), growth hormone (gh) and insulin-like growth factor (igf1). The transition between endogenous and exogenous feeding stage is indicated by shades of blue. Values represent means (± SEM), while different letters represent significant differences at alpha = 0.05.

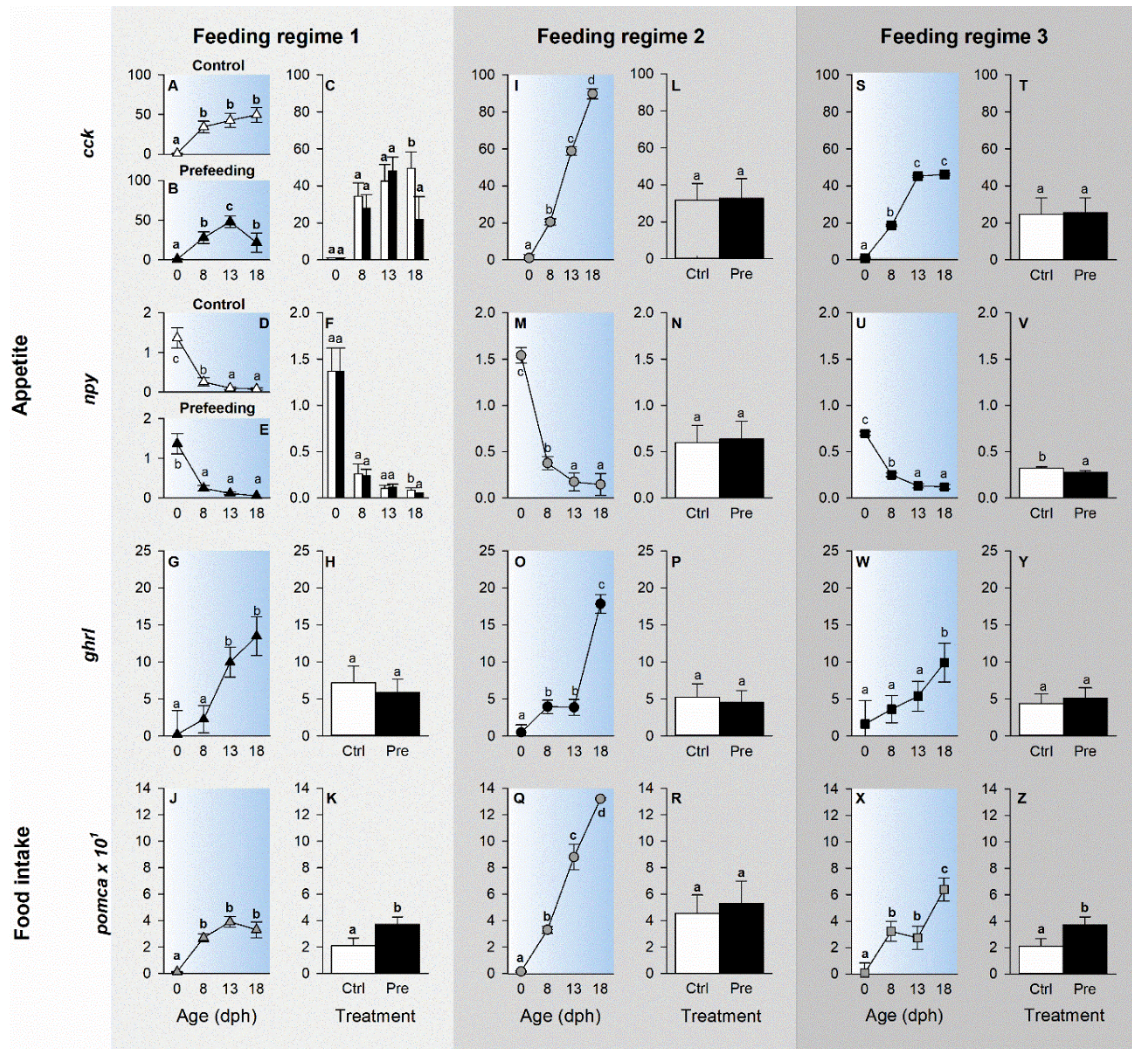

Figure 5. Effect of age in days post hatch (dph) and prefeeding treatment (control (Ctrl) vs. prefeeding (Pre)) on European eel, *Anguilla anguilla* larval appetite and food intake related gene expression in three different feeding regimes (1, 2 and 3). Relative expression of cholecystokinin (*cck*) neuropeptide Y (*npy*), ghrelin (*ghrl*) and proopiomelanocortin a (*pomca*). The transition between endogenous and exogenous feeding stage is indicated by shades of blue. Values represent means (± SEM), while different letters represent significant differences at alpha = 0.05.

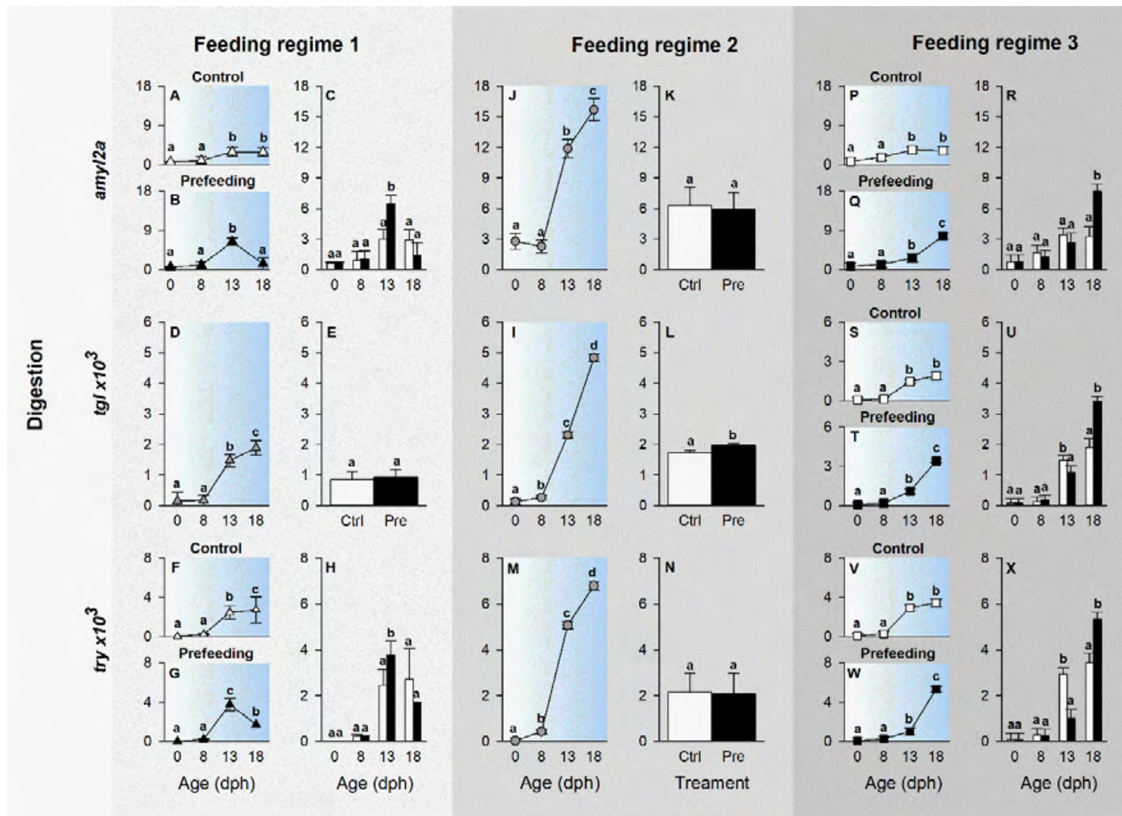

Figure 6. Effects of age in days post hatch (dph) and prefeeding treatment (control (Ctrl) vs. prefeeding (Pre)) on European eel, *Anguilla anguilla* larval digestion related gene expression in three different feeding regimes (1, 2 and 3). Relative expression of amylase (*amyl2a*), triglyceride lipase (*tgl*) and trypsin (*try*). The transition between endogenous and exogenous feeding stage is indicated by shades of blue. Values represent means (± SEM), while different letters represent significant differences at alpha = 0.05.

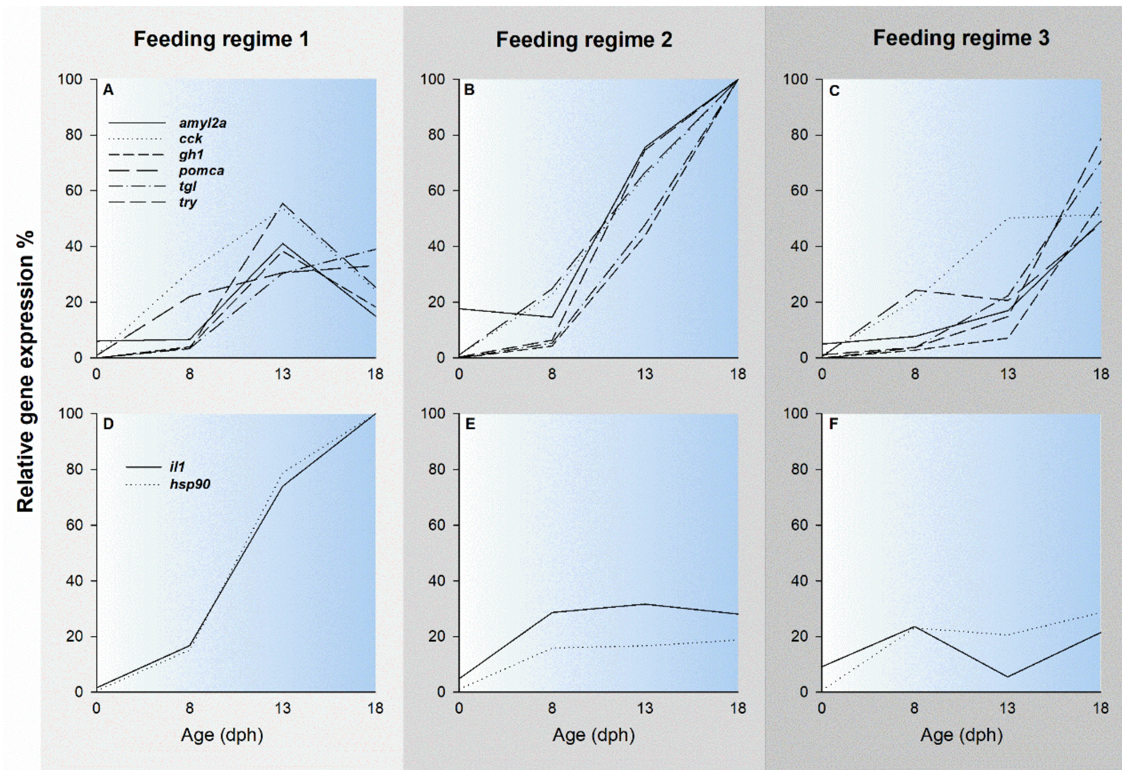

Figure 7. Standardized expression of genes related to digestion, appetite, food intake and growth (A, B and C) as well as immune and stress response (D, E and F) in three different feeding regimes for prefed European eel, *Anguilla anguilla* larvae. Expression was calculated in relation to the highest mRNA level (Feeding regime 2) and expressed in percentage. The transition between endogenous and exogenous feeding stage is indicated by shades of blue.

Study 4:

First-feeding regimes for European eel larval culture: insights at morphological, nutritional, and molecular levels

Benini, E., Bandara, K., Politis, S.N., Engrola, S., Sørensen, S.R., Nielsen, A., Conceição, L.E.C., Tomkiewicz, J.

Manuscript

First-feeding regimes for European eel larval culture: insights at morphological, nutritional, and molecular levels

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Abstract

The life cycle of European (*Anguilla anguilla*) eel has not been closed yet in captivity and the lack of knowledge regarding nutritional requirements during the first-feeding window has hindered the development of sustainable aquaculture. Previous feeding attempts using rotifer paste, resulted in no larval survival beyond the first-feeding stage. In this study, three new experimental diets were formulated and tested on hatchery produced European eel larvae, throughout and beyond the first feeding window (from 9 to 28 days post hatch (dph)). The composition of Diet 1 followed the Japanese eel (*Anguilla japonica*) larval recipe, while Diets 2 and 3 represent modifications, targeting different sizes of dietary proteins (3 kDa *vs.* 10 kDa). Results showed that eel larvae successfully ingested all three diets, with feeding incidence increasing from 37.8% to 54.4%, while gut fullness increased from 20.03% to 24.9% between 15 and 22 dph, whereas highest gut fullness was registered for Diet 3. Between 9 and 15 dph larvae fed Diet 1 had an improved survival, morphology (length and body area) and gene expression (*amyl2a*, *tgl*, *pomca* and *gh*). At the same time, two periods of high mortality were identified. The first, was registered shortly after the first introduction of feed (10-12 dph), which was probably driven by the challenging transition from endogenous to exogenous feeding processes. The second, occurred at 20-24 dph, probably indicating that eel larvae reached the "*point of no return*", which is further supported by the continuous upregulation (peaking at 22 dph) of *ghrl*, the gene encoding the "*hunger hormone*", indicating that most eel larvae were fasting. Interestingly though, larvae fed Diet 3, downregulated the expression of *ghrl* again beyond 22 dph, probably demonstrating that those larvae successfully overcame the challenges during this period, resulting in improved survival and growth (length, body area and dry weight) beyond the "*point of no return*", compared to larvae fed the other diets. Moreover, the upregulated expression patterns of the genes encoding the major digestive enzymes (*try*, *tgl*, and *amyl2a*) at 22 dph in larvae fed Diet 3,

demonstrate that this benefit occurred also on the molecular level. Furthermore, the expression patterns of genes related to digestion (*try*, *tgl*, *amyl2a*), food intake (*pomca*), and growth (*gh*) continued to increase towards 28 dph for larvae fed Diet 3, underlining the success of this diet and indicating an improving maturity and functionality of the molecular feeding, digestion, and growth mechanisms. Overall, we observed an early benefit of Diet 1, but it seems that the inclusion of more complex dietary proteins (Diet 3), but not hydrolysed peptides (Diet 2) is beneficial in order to promote larval ontogenetic performances after the successful transition to exogenous feeding. We conclude that the digestive and assimilation capacity of European eel larvae may vary throughout ontogeny and consequently, the dietary regime might need to be adapted according to stage specific nutritional requirements and preferences. However, further research is needed to improve larval feeding and rearing techniques, but the results of the present study provide an unprecedented step towards establishing a first feeding culture of hatchery produced European eel larvae and a basis for future research efforts towards sustainable aquaculture of this critically endangered species.

Key words

Anguilla anguilla, hatchery, diet composition, gene expression, growth, appetite, digestion

Highlights

- Two periods of high mortality were identified at 10-12 dph, shortly after first introduction of feed and at 20-24 dph, marking the point of no return.
- During first-feeding (15 dph), Diet 1 initially improved survival and resulted in bigger larvae with upregulated digestion, food intake and growth related genes.
- Beyond the first-feeding window (22 dph), Diet 3 resulted in higher survival, growth (dry weight, length and body area) and higher gut-fullness.
- Larvae fed Diet 3, showed upregulated digestion (*try*, *tgl*, *amyl2a*), food intake (*pomca*), and growth (*gh*) related genes at 22 dph, which continued to increase towards 28 dph.

1. Introduction

Closing the life cycle in captivity of European eel is a necessary requirement to develop sustainable aquaculture of this species. Due to the decline of the population, together with low levels of glass eel recruitment, this species is critically endangered and subject to trade restrictions (ICES, 2021; Pike et al., 2020). Thus, hatchery production of offspring is essential in order to lift limitations on aquaculture production and markets for European eel (Tomkiewicz, 2019). Nonetheless, the development of hatchery protocols is challenged by lack of knowledge regarding the species biology, especially the long and complex larval stage (Righton et al., 2021). As such, in nature, the European eel larval stage duration can be up to 28 months, during which, they drift over thousands of kilometres from the spawning area, located in the oligotrophic waters of the Sargasso Sea (Munk et al., 2010), towards the European and North African coasts. Eel larvae, called leptocephali, are very peculiar as they are characterized by a laterally compressed body, shaped as a leaf, and mostly transparent due to the accumulation of glycosaminoglycan (GAG), used to store energy and provide structural support (Pfeiler, 1999; Pfeiler et al., 2002). After the larval stage, they metamorphose into glass eels, then into elvers, exploring habitats in estuarine or freshwater environments, where they grow to sexually immature yellow eels. Subsequently, eels initiate the so called "silvering process" and undertake a catadromous migration back to the Sargasso Sea to spawn and complete their life cycle (Tesch, 2003). Nevertheless, information regarding the eel early life stages is scarce, which has considerably delayed the development of culture techniques and technologies, which relies only on targeted experimental research and analytical techniques.

At present, the main bottleneck for progressing in rearing hatchery-produced eel larvae is the establishment of an adequate feeding regime (Ijiri et al., 2011). In this regard, several studies have aimed to determine the natural diet of anguilliform

leptocephali, where initially, larval guts appeared mostly empty or contained an undefined substance (Hulet and Robins, 1989; Pfeiler, 1986). Thereafter, it was proposed that particulate organic matter (POM) is the main source of eel larval nutrition, possibly in the form of marine snow, larvacean houses, and/or zooplankton fecal pellets (Mochioka and Iwamizu, 1996; Otake et al., 1993). This result has been confirmed by recent studies using advanced molecular techniques (Ayala et al., 2018). Interestingly though, using analyses of the bulk nitrogen stable isotope (Miller et al., 2013), revealed that the natural feed source of Japanese eel (*Anguilla japonica*) larvae is biochemically far from the diet used to successfully rear them in captivity.

The initial breakthrough diet used to culture Japanese eel, was based on egg yolk of spiny dogfish, *Squalus acanthias* (Tanaka et al., 2001), allowing the larvae to survive up to 26 days post hatch (dph) and to reach a total length of 8.67 mm (Kagawa et al., 2005). Further modifications of that diet, by adding soybean peptides, a mix of vitamins and minerals, as well as krill extract resulted in improved growth (22 mm in length) and survival up to 100 dph (Tanaka et al., 2003). Subsequent diets, replacing soybean peptides with chicken egg albumin peptides and chitin oligosaccharides, improved the survival and growth of Japanese eel larvae, while reducing the time of metamorphosis into glass eels to ~131 dph (Okamura et al., 2009). However, the use of liquid diets, containing spiny dogfish egg yolk, leads to rapidly deteriorating water quality in the rearing tanks (Tanaka, 2015). Therefore, to ensure restoration of water quality, feeds need to be flushed away after each feeding event and tanks replaced daily (Okamura et al., 2020). Consequently, due to all these improvements, the production of Japanese eel has quickly progressed, allowing the closure of the life cycle in captivity and the production of second generations of artificially reared larvae (Tanaka, 2015). Thus, thanks to the enhancements related to the feeding and rearing techniques for

leptocephali and glass eel, the production and commercialization of artificially produced Japanese eel will be possible in the near future.

Regarding European eel, it has been observed that larvae can successfully ingest a paste made of rotifers, *Brachionus plicatilis* (Butts et al., 2016). Here, despite successful initiation of first-feeding at 15 dph (cultured at 18°C) and an improved body area compared to starving conspecifics, the larvae did not survive past 24 dph (Politis et al., 2018a). This indicates the low suitability of this diet or at least the low palatability for larvae, which probably entered the so called "point of no return", where larvae that did not timely initiate feeding, are not able to digest food or assimilate nutrients and eventually perish (May, 1974; Sifa and Mathias, 1987). Interestingly though, genes encoding the major digestive enzymes were expressed earlier than the actual initiation of first feeding (Politis et al., 2018a), indicating a potential earlier maturation of the gastro intestinal tract. In this regard, probiotic and prebiotic products have been applied to explore gut-priming strategies (Politis et al., Study 2), while pre-feeding protocols were explored as a strategy to promote feeding initiation and thus, transition towards exogenous feeding (Benini et al., subm., Study 3). Here, this study indicated that early availability of dietary nutrients can influence the molecular ontogeny of feeding related mechanisms and processes, but the successful initiation of larval feeding and growth ultimately depends on the quality and palatability of the diet(s).

The present study combined insights regarding feed development and feeding regimes, gained from European and Japanese eel research, to formulate three diets as potential first feeding prototypes for hatchery produced European eel larvae. The diets were tested experimentally from the onset of the first feeding stage at 10 dph until 28 dph. Larval mortality was recorded daily while sampling was conducted at regular intervals to obtain larval biometrics and gene expression patterns to follow the molecular ontogeny of digestive functions and growth of the larvae during the experimental period.

Genes studied were selected to target mechanisms and processes, such as appetite [ghrelin (*ghrl*)], food intake [proopiomelanocortin (*pomc*)], digestion [trypsin (*try*), triglyceride lipase (*tgl*), amylase (amyl2a)], energy metabolism [cytochrome-c-oxidase (*cox1*)], and growth [growth hormone (*gh*)].

2. Materials and methods

2.1 Ethic 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). Eels 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, Sigma Aldrich, Germany). Larvae were anesthetized and euthanized using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany) at a concentration of 7.5 and 15 mg/L, respectively.

2.2 Broodstock management, gamete production and offspring culture

Female broodstock included silver eels caught from a brackish lake, Saltbæk Vig (Zealand, Denmark), while male eels were obtained from a commercial eel farm (Royal Danish Fish, Hanstholm, Denmark). Once transported to the EEL-HATCH facility (Hirtshals, Denmark), the broodstock was acclimatized for three weeks before assisted reproduction protocols were applied to initiate gametogenesis, as described in Kottmann et al. (2020). In females, weekly injections of salmon pituitary extract (Argent Chemical Laboratories, Washington, USA) at a dose of 18.75 mg/kg initial body weight was necessary to induce vitellogenesis, while final maturation was induced using 17a,20b-dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma-Aldrich Chemie, Steinheim,

Germany) according to da Silva et al. (2018). On the other hand, weekly injections of human chorionic gonadotropin were necessary to induce spermatogenesis in male broodstock (Sigma-Aldrich, Missouri, USA) at 1.5 IU/g initial body weight according to Koumpiadis et al. (2021).

Gametes were strip-spawned and fertilized at 20°C, using a standardized sperm to egg ratio with gamete contact time of 5 min (Butts et al., 2014; Sørensen et al., 2016). Here, pooled milt from three males was used to fertilize the batch of eggs (Benini et al., 2018). Thereafter, incubation followed the protocol described by Politis et al., (2018a), where temperature was lowered to 18°C (Politis et al., 2017) and light kept below ~10 lx (Politis et al., 2014). Hatching occurred at ~56 hours post fertilization in the incubators. Subsequently, the newly hatched larvae were transferred into 77 L tanks, connected to a 1.7 m^3 recirculating aquaculture system (RAS), composed of a biofilter (RK elements, 750 m^2 RK BioElements, Skive, Denmark), a trickle filter (BioBlok 200, EXPO-NET, Hjørring, Denmark), a protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic GmbH, Bissendorf, Germany) and UV light (11 W, JBL ProCristal, Neuhofen, Germany). Temperature was maintained at 18-20°C and salinity at ~36 psu (Politis et al., 2017; 2018b; 2021). Water flow was set to 600 mL/min, while light regime was set to constant darkness (Politis et al., 2014).

2.3 Selection of larval batches for the experiment

A batch of larvae was selected based on hatching success (75.8%) and number of hatched larvae (54666 larvae). Hatching success was calculated as described in Benini et al. (2022a), where subsamples of ~ 100 embryos were randomly collected from the incubators at 48 hpf. The embryos were inserted into replicated 200 mL sterile tissue culture flasks (VWR, Denmark) filled with seawater, including rifampicin and ampicillin (each 50 mg/L, Sigma-Aldrich, Missouri, USA) to counteract microbial interference

(Sørensen et al., 2014). App. 12 h after incubation, the numbers of hatched larvae versus unhatched or dead embryos in each flask were recorded and hatching success calculated as number hatched versus the initial total stocking number. Female total length and weight were 66 cm and 570 g, respectively. Body length and weight (mean ± SD) of males (n = 3) were 42.7 ± 2.1 cm and 136.5 ± 12.23 g, respectively.

2.3 - Experimental design and first-feeding regimes

When reaching the first-feeding stage, at the end of the 9th dph, eel larvae were divided into replicated Kreisel tanks (n = 9) of 8 L capacity each, at a density of ~60 larvae/L and randomly connected to two separate, but identical 0.65 m^3 RAS units (similar to the described above). Here, salinity was constantly kept at ~18 psu (Politis et al., 2021, 2018b), temperature at ~20°C (Politis et al., 2017), and light (~500 lux) was only turned on during feeding (Butts et al., 2016; Okamura et al., 2019). Flow rates were kept at ~ 420 mL/min.

In each Kreisel tank, larvae received feed at a concentration of 0.5 mL diet per L of water five times per day at two hour intervals. Here, light was turned on and water current was stopped to give larvae the possibility to feed on the diet, which was directly pipetted on the bottom of the tank. Larvae were allowed to feed for 30 min, after which light was turned off and water flow on again. The remaining feed was flushed away, using a gentle jet of water. Thereafter, Kreisel tanks were disconnected from the corresponding RAS units, so that the rearing water was allowed to run out of the tank for 30 min. Thus, new clean water, pre-adjusted to 20°C and 18 psu, was used to refill each RAS unit. Larvae were moved into clean tanks daily (Benini et al., subm., Study 3; Okamura et al., 2014).

2.4 - Composition of diets

Three diets were formulated and tested in this study. All diets were freshly prepared daily. The biochemical composition of all diets is described in Table 1 and composition of specific fatty acids in Table 2. All diets were adjusted to reach a similar liquid paste-like consistency using reverse osmosis water. The composition of Diet 1 resembled the larval Japanese eel recipe as described in Tanaka et al. (2001, 2003), containing spiny dogfish (*Squalus acanthias*) eggs, krill extract and soybean peptide of 99.9 % purity (Sgonek Biological Technology Co. Ltd, China). The krill extract consisted of thawed, deskinned krill (Akudim, Esbjerg, Denmark), mixed with reverse osmosis water at a 1:2 ratio, sieved through a sock net (0.2 mm mesh size) and pasteurized in the oven for 30 min at 60°C. From a composition point of view, Diet 1 contained less protein and more lipids compared to the other diets. Diets 2 and 3, represent modifications of Diet 1. Those two diets had a proximal composition of 60% protein and 30% lipid, but the protein source and molecular size differed. Here, Diet 2 contained fish hydrolysates (Diana-Aqua, France), which consisted of peptides of approximately 3 kDa in size. Fish protein hydrolysates are obtained from the hydrolysis process of fish and crustacean processing by-products and are characterized by a high content of peptides and free amino acids as compared to the composition of native protein (Aguila et al., 2007). In comparison, Diet 3 contained whey (Volacactive UltraWhey 80 Instant, Volac International Ltd, Hertfordshire, UK), with a molecular protein weight ranging between 10 and 12 kDa. Whey is a by-product of cheese making or casein in dairy production (Amer et al., 2019). The fatty acid composition was relatively similar among diets.

Table 5 – Proximal composition of three experimental diets used to feed European eel (Anguilla anguilla) larvae.

	Diet 1	Diet 2	Diet 3
Dry mater (%)	27.1 ± 0.12	29.8 ± 0.09	36.01 ± 0.10
Protein (%)	50.89 ± 0.35	61.12 ± 0.17	59.09 ± 0.81
Lipid (%)	37.52 ± 0.25	27.49 ± 0.10	27.51 ± 0.28
Ash (%)	3.33 ± 0.04	3.21 ± 0.12	2.91 ± 0.03
Energy (kj/g)	29.30 ± 0.18	27.93 ± 0.17	28.46 ± 0.18

Table 6 – Fatty acids of three experimental diets used to feed larvae of European eel, Anguilla anguilla. Each class of fatty acids is expressed in relation to the amount of total lipid (mg FA/mg lipid).

	Diet 1	Diet 2	Diet 3
14:0	1.82	2.65	2.68
15:0	0.83	0.85	0.96
16:0	85.97	92.51	94.08
18:0	24.80	24.95	26.29
24:0	5.07	6.27	5.39
Σ SFA	118.49	127.25	129.41
14:1	0.00	0.00	0.00
16:1	11.77	12.73	11.58
18:1n-9	114.51	114.81	113.89
20:1	42.82	37.11	42.21
22:1	13.67	8.31	12.38
24:1	4.04	3.95	3.94
Σ MUFA	186.82	176.92	184.01
18:2n-6	9.00	9.25	8.50
18:3n-6	2.30	1.32	1.83
20:2n-6	0.00	0.00	0.00
20:3n-6	0.00	0.00	0.00
20:4n-6	18.53	19.01	18.78
Σ (n-6) PUFA	29.83	29.58	29.10
18:3n-3	2.35	2.77	1.84
18:4n-3	2.15	2.37	2.57
20:4n-3	3.19	3.70	3.48
20:5n-3	55.10	53.13	63.86
22:5n-3	18.89	20.85	19.91
22:6n-3	104.76	105.57	101.29
Σ (n-3) PUFA	186.44	188.39	192.96
Σ PUFA	216.27	217.97	222.06
Σ FA	555.59	546.89	565.80

2.5 - Larval survival and biometry

From 10 dph and onwards, dead larvae were counted and removed daily from all experimental units. Larval cumulative mortality was calculated as a percentage from 10 until 28 dph.

Moreover, subsamples (n = 3) of 10 larvae each were collected at hatch (0 dph) and larvae photographed, for measuring initial larval biometry. Thereafter, pools of 10 larvae per replicated tank (*n* = 3) and per diet (*n* = 3) were sampled and photographed at selected developmental stages, such as before introduction of the experimental diets (9 dph), the middle of first-feeding window (15 dph), at the end of the first-feeding window (22 dph) and beyond the first-feeding stage (28 dph). All larvae were anesthetized using tricaine methanesulfonate (MS-222; Sigma-Aldrich, Missouri, USA) prior to digital imaging and euthanized post-sampling by using an MS-222 overdose. All images were taken using a digital camera (Digital Sight DS-Fi2, Nikon Corporation, Japan) attached to a zoom stereomicroscope (SMZ1270i, Nikon Corporation, Japan), while NIS-Elements D analysis software (Version 3.2) was used to assess larval standard length and body area from the images (Nikon Corporation, Japan). Feeding incidence and gut fullness at 15 and 22 dph was calculated as described in Butts et al. (2016).

2.6 - Dry weight

At 9, 15 and 22 dph, 10 larvae per replicated tank (*n* = 3) and per diet (*n* = 3) were euthanized using MS-222, directly frozen at -80°C and, thereafter, freeze-dried using a laboratory freeze/dryer (Christ Beta 2, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Freeze-dried larvae were weighted, using a microbalance (Mettler-Toledo A/S, Denmark). The individual dry weight was calculated by dividing the dry weight of each larval pool by the number of larvae in the sample.

2.5 - Gene expression

For molecular analysis, larvae were sampled before introduction of feeding (0 and 9 dph) and throughout the first-feeding window (15 and 22 dph). At 28 dph, there were not enough larvae left in Diets 1 and 2 to perform molecular analyses. Therefore gene expression was conducted only for larvae fed Diet 3. Approximately 10-15 larvae were euthanized using MS-222, rinsed with deionized water, preserved in a RNA later (Stabilization Reagent) and kept at -20°C following the procedure suggested by the supplier (Qiagen, Hilden,Germany). RNA was then extracted using the NucleoSpin RNA Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. RNA concentration (110 ± 43 ng/ml) and purity (260/280 = 2.09 ± 0.03, 230/260 = 2.02 ± 0.12) were determined by spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany) and normalized to a common concentration of 100 ng/ml with HPLC water. From the resulting total RNA, 450 ng were transcribed using the qScript^{TM} cDNA Synthesis Kit (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipe out step prior to transcription [PerfeCtaR DNase I Kit (Quantabio, Germany)].

The expression levels of seven target and three reference (housekeeping) genes were determined by quantitative real-time PCR (qRT-PCR), using specific primers. Primers were designed using Primer3 software v 0.4.01, based on cDNA and predicted cDNA sequences available in Genbank databases (Table 3). All primers were designed for an amplification size ranging from 75 to 200 nucleotides. The elongation factor 1 a (*ef1a*), 40S ribosomal S18 (*rps18)* and tubulin β1 (*tubβ*) genes were chosen as housekeeping genes, as they have also been suggested to be the most stable in fish larvae and thus, the most reliable reference genes (McCurley and Callard, 2008). Their stability was statistically confirmed, where their expression was not significantly different across treatments.

Expression of genes in each larval sample of each tank (*n* = 3), diet (*n* = 3) and age (n = 5) were analyzed in three technical replicates using the qPCR Biomark^{TM} HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips). In brief, a pre-amplification step was performed with a 500 nM primer pool of all primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 mL cDNA per sample for 10 min at 95°C; 14 cycles: 15 sec at 95°C and 4 min at 60°C. Obtained PCR products were diluted 1:10 with low EDTA-TE buffer. The pre-amplified 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 mM. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60°C. The relative quantity of target gene transcripts was normalized and measured using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Coefficient of variation (CV) of technical replicates was calculated and checked to be < 0.04 (Hellemans et al., 2007).

Table 7 - Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR. Primers were designed based on sequences available on Genbank databases.

Full Name	***Function***	***Abbreviation***	***Primer sequence (5' 3') Forward***	***Primer sequence (5' 3') Reverse***	***Accession Number***
Elongation factor 1	Reference	*ef1*	CTGAAGCCTGGTATGGTGGT	CATGGTGCATTTCCACAGAC	XM_035428800.1
18s Ribosomal RNA	Reference	*rps18*	ACGAGGTTGAGAGAGTGGTG	TCAGCCTCTCCAGATCCTCT	XM_035428274.1
Tubulin β	House keeping	*tubβ*	TGATGAGCACGGTATTGACC	TGGCACATACTTTCCACCAG	XM_035419873.1
Growth hormone	Growth	*gh*	GTTTGGGACCTCTGATGGGA	AGCAGGCCGTAGTTCTTCAT	XM_035398906.1
Cytochrome-C-Oxidase	Energy	*cox1*	CTACTCCTCTCCCTGCCAGT	CTTCTGGGTGGCCGAAGAAT	YP_163818.1
Prepro-Ghrelin	Appetite	*ghrl*	TCACCATGACTGAGGAGCTG	TGGGACGCAGGGTTTTATGA	XM_035381207.1
Proopiomelanocortin v1 and v2	Food intake	*pomca*	GCCTGTGCAAGTCTGAACTG	GACACCATAGGGAGCAGGAA	XM_035421304.1
Amylase	Digestion	*amyl2a*	AGACCAACAGCGGTGAAATC	TGCACGTTCAAGTCCAAGAG	XM_035420193.1 v3
Triglyceride lipase	Digestion	*tgl*	CTGACTGGGACAATGAGCGT	CGTCTCGGTGTCGATGTAGG	XM_035399731.1
Trypsin	Digestion	*try*	CTGCTACAAATCCCGTGTGG	GGAGTTGTATTTGGGGTGGC	XM_035429595.1

2.7 Statistical analysis

All data were analyzed using R studio statistical analysis software (Version 1.3.959, *RStudio: Integrated Development for R. RStudio, PBC, Boston, MA*). Residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted values) to ensure they met model assumptions. Data were log (10) transformed to meet these assumptions when necessary. Alpha was set at 0.05 for testing main effects and interactions. Treatment means were contrasted using Tukey's Honest Significant Difference test. Standard length, body area, dry weight as well as gene expression (8 genes) at each age (0, 9, 15, 22 and 28 dph) were analyzed using a series of mixed model ANOVAs (PROC GLM). The main model variables were treatment (diet 1 *vs.* diet 2 *vs.* diet 3) and age (0, 9, 15, 22 and 28 dph), while replicated tanks were considered random. The initial model tested included an interaction effect between treatment and age. The model was reduced when possible. The final model was validated through analyses of the residuals. As for survival and daily mortality, the interaction effect between treatments and age was tested and a series of pairwise t-tests were run at all developmental stages (from 10 to 27 dph).

Results

3.1 Biometry

3.1.1. Length

Larval standard length (SL) was measured at hatch (0 dph) and throughout development (at 9, 15, 22 and 28), where it was significantly ($p < 0.01$) influenced by the treatment × age interaction. The model was then decomposed into a series of reduced ANOVA models to determine the age for each diet (Fig. 1A, B and C) and the effect of diets for each age (Fig. 1D). Larval length significantly ($p < 0.01$) increased from 3.83 ± 0.084 to 6.59 ± 0.089 mm from hatch until 9 dph (Fig. 1A, B and C). During the feeding period, larvae fed Diet 1 significantly ($p < 0.001$) grew between 9 and 15 dph, while no

difference in length was observed between 15 and 22 dph. For larvae fed Diet 2, no increase in SL was noted between 9 and 22 dph, while a significant ($p < 0.01$) decrease was detected between 22 and 28 dph. In larvae fed Diet 3, a significant ($p < 0.001$) increase in SL was detected between 9 and 15 dph, while hereafter, larval SL remained stable until the end of the experiment (28 dph). Moreover, the dietary treatments influenced larval length during the feeding period, where as evident in Fig. 1D, significantly ($p < 0.01$) longest larvae at 15 dph were the ones fed Diet 1, while at 22 and 28 dph, significantly ($p < 0.01$) longest larvae were the ones fed Diet 3.

3.1.2. Body area

Body area, measured at hatch (0 dph) and throughout larval ontogeny (at 9, 15, 22 and 28), was significantly ($p < 0.001$) influenced by the treatment × age interaction. Therefore, the model was decomposed into a series of reduced ANOVA models to determine the effect of age for each diet (Fig. 1E, F and G) and the effect of diets for each age (Fig. 1H). Here, as expected, all larvae significantly ($p < 0.001$) grew larger from hatch until 9 dph (Fig. 1E, F and G). Thereafter, the body area of larvae fed Diet 1 significantly ($p < 0.01$) increased between 9 and 15 dph, while a decreasing trend was registered on 22 dph. Similarly, for larvae fed Diet 2 no change was noted between 15 and 22 dph, but a significant ($p < 0.01$) decrease in body area was observed on 28 dph. On the other hand, for larvae fed Diet 3, we detected a significant ($p < 0.01$) increase in body area between 9 and 15 dph, while hereafter, larval body area remained stable until the end of the experiment (28 dph). Significant differences in body area between treatments were observed at 15 dph, where larvae fed Diet 1 had a significantly ($p < 0.001$) larger body area compared to Diet 2, while at 22 and 28 dph, Diet 3 fed larvae had a significantly ($p < 0.0001$) larger body area compared to larvae from the other treatments (Fig. 1H).

3.2 Survival

Survival (%) was significantly ($p < 0.001$) affected by the age × treatment interaction. Thus, the model was decomposed into a series of reduced one-way ANOVAs to determine the effect of treatment for each age (Fig. 2 A,B,C) and the effect of age for each treatment (Fig. 2D). Overall, the survival rate significantly decreased with development, however, between 13 and 19 dph, it remained stable in all three treatments (Fig. 2A, B and C). Moreover, until 20 dph, the survival rate was highest in Diet 1, while beyond 21 dph, the highest survival rate was observed in Diet 3 (Fig. 2D). At 28 dph, we observed a survival of 4 % for larvae fed Diet 3 and only 0.5 % for larvae fed Diet 2, while no larvae survived when fed Diet 1 (Fig. 2D).

3.3 Feed incidence and growth rate

Feeding incidence (%) did not show any difference between dietary treatments (Fig. 3A), however it significantly ($p < 0.001$) increased from 37.8 ± 4.26 to 54.4 ± 4.38 between 15 and 22 dph (Fig. 3B). Moreover, gut fullness (%) was affected by both, diet (Fig. 3C) and age (Fig. 3D), where it significantly ($p < 0.05$) increased throughout development and was significantly ($p < 0.02$) higher in larvae fed Diet 3 compared to Diets 1 and 2.

3.4 Dry weight

Larval dry weight, measured at 9, 15 and 22 dph, was significantly ($p < 0.001$) affected by the treatment × age interaction. When the model was decomposed to detect the effect of treatment at each age, we could observe no statistically significant difference between treatments at 15 dph, while at 22 dph larvae fed Diet 3 had a significantly ($p = 0.0087$) higher dry weight compared to larvae fed Diet 2 (Fig. 4D). Moreover,

irrespectively of the diet used, the larval dry weight significantly ($p < 0.01$) decreased throughout development (Fig. 4A, B and C).

3.5 Gene expression

3.5.1 Digestion genes

In this study, the expression of genes related to digestion (*amyl2a, tgl, try*) were significantly ($p < 0.01$) affected by the treatment × age interaction (Fig. 5). Thus, the model was decomposed into a series of reduced one-way ANOVAs to determine the effect of age for each treatment and the effect of treatment for each age.

The expression of *amyl2a* significantly ($p < 0.001$) increased approximately 5-fold between hatch and 15 dph in all three treatments. However, beyond 15 dph it significantly decreased ($p < 0.01$) for larvae fed Diet 1 and 2 (Fig. 5A and B), while the expression of *amyl2a* significantly increased ($p < 0.0001$) for larvae fed Diet 3, reaching peak values on 28 dph (Fig.5C). Moreover, we observed that the expression of *amyl2a* was significantly ($p < 0.01$) highest in Diet 1 at 15 dph, while significantly ($p < 0.01$) highest in Diet 3 at 22 dph (Fig. 5D).

Similarly, the expression of *tgl* significantly ($p < 0.01$) increased more than 1000-fold between 0 and 15 dph. However, after this point we observed that the expression of *tgl* significantly ($p<0.001$) decreased for larvae fed Diet 1 (Fig. 5E) and remained stable for larvae fed Diet 2 (Fig. 5F), while significantly ($p < 0.01$) increased for larvae fed Diet 3, reaching peak values on 28 dph (Fig. 5G). As evident in Figure 5H, expression of *tgl* was significantly ($p < 0.01$) highest for larvae fed Diet 1 at 15 dph, while significantly ($p < 0.01$) highest for larvae fed Diet 3 at 22 dph.

In addition, the expression of *try* followed a similar pattern as for *amyl2a* and *tgl,* significantly ($p < 0.001$) increasing approximately 50-fold towards 15 dph. However,

expression of try significantly decreased ($p < 0.001$) for larvae fed Diets 1 and 2 (Fig. 5I and J), while it significantly ($p < 0.01$) increased for larvae fed Diet 3 (Fig.5K). Moreover, while no significant difference was observed at 15 dph, the expression of try was significantly ($p < 0.01$) higher in larvae fed Diet 3 compared to the other experimental groups (Fig. 5L).

3.5.2 Appetite and food intake genes

The expression of genes regulating appetite and food intake were significantly ($p < 0.01$) affected by the treatment × age interaction. Thus, the model was decomposed into a series of reduced one-way ANOVAs to determine the effect of age for each treatment (Fig. 6A-C and E-G) and the effect of treatment for each age (Fig. 6D and H).

The expression of the gene encoding ghrelin (*ghrl*) significantly ($p < 0.01$) increased 20-fold from hatch until 22 dph (Fig 6A, B and C) and significantly ($p < 0.01$) decreased beyond this point for larvae fed Diet 3 (Fig. 6C). Moreover, at 9 and 15 dph, no significant difference in *ghrl* expression was observed between treatments. However, at 22 dph, larvae fed Diet 1 significantly ($p < 0.05$) downregulated *ghrl* expression compared to Diet 2 (Fig. 6D).

The expression of *pomca* significantly ($p < 0.01$) increased approximately 50-fold until 15 dph in all diets (Fig. 6E, F and G). Beyond that point, *pomca* expression significantly ($p < 0.01$) decreased for larvae fed Diet 1 and 2 (Fig. 6E and F), while it remained constant between 15 and 22 dph for larvae fed Diet 3, but was significantly ($p < 0.01$) upregulated thereafter, with an overall 80-fold increase towards the end of the experiment (28 dph) compared to hatch (Fig. 6G). Moreover, at 15 dph the expression of *pomca* was significantly ($p < 0.01$) highest in larvae fed Diet 1, while no difference between treatments was observed at 22 dph (Fig. 6H).

3.5.3 Growth and energy

The expression of *gh* (growth) and *cox1* (energy metabolism) were significantly ($p < 0.01$) affected by the treatment × age interaction. Thus, the model was decomposed into a series of reduced one-way ANOVAs to determine the effect of age for each treatment (Fig. 7A-C and E-G) and the effect of treatment for each age (Fig. 7D and H).

Overall, *gh* expression significantly ($p < 0.01$) increased until 15 dph. However, beyond 15 dph, *gh* expression decreased in larvae fed Diet 1 (Fig. 7A), while it remained constant for Diet 2 (Fig. 7B). On the contrary, *gh* expression significantly ($p < 0.01$) and progressively increased beyond 15 dph for larvae fed Diet 3 (Fig. 7C). Moreover, at 15 dph the expression of *gh* was significantly ($p < 0.01$) highest in larvae fed Diet 1, while at 22 dph it was significantly ($p < 0.01$) highest for larvae fed Diet 2 and 3 (Fig. 7D). Markedly, in Diet 3, the expression of *gh* reached peak values on 28 dph, approximately 2-fold higher compared to 22 dph and approximately 300-fold upregulated compared to hatch (Fig. 7C and D).

The expression of *cox1* remained constant throughout the experimental period for larvae fed Diet 1 and 3 (Fig. 7E and G), while it was significantly ($p < 0.001$) upregulated on 15 and 22 dph for larvae fed Diet 2 (Fig. 7F). As such, at 15 and 22 dph, the expression of *cox1* was significantly ($p < 0.01$) higher in Diet 2 fed larvae compared to the other experimental groups (Fig. 7H).

4. Discussion

The establishment of first feeding in larval culture is currently the major challenge developing European eel hatchery techniques for a sustainable closed-cycle aquaculture production. Here, together with advancements in culture systems and technology, identification of suited diets and nutritional requirements is pivotal. In particular, during the first-feeding stage, the quality of feed has a major impact on the development of

European eel larvae (Benini et al., subm., Study 3; Butts et al., 2016; Politis et al., 2018a). The available knowledge regarding natural feeding preferences during the European eel larval stage is based on analyses of gut content, where gelatinous plankton has been suggested to be the main feed source (Ayala et al., 2018; Mochioka and Iwamizu, 1996; Riemann et al., 2010). Further information has been collected by studying the feeding behaviour of European eel larvae in culture, identifying the importance of light during feeding and of the consistency/liquidity of the diets (Butts et al., 2016; Politis et al., 2018a). Thereafter, experimental diets were developed to test first-feeding processes in European eel larvae, improving survival rate and providing first knowledge on the digestive capacity during this stage (Benini et al., subm., Study 3, Politis et al., 2018a). Those aforementioned studies, paved the way for expanding research efforts towards formulating potential suitable diets for first-feeding European eel larvae. As such, in the present study, three diets (1, 2 and 3) were formulated and tested. Diet 1 imitated the diet used to feed Japanese eel larvae (Okamura et al., 2012), while the other Diets represent modifications of Diet 1, aiming to either ease nutrient availability by the addition of fish hydrolysate (Diet 2) or increasing the molecular size of dietary protein by the addition of whey (Diet 3).

Overall, eel larvae successfully ingested all diets tested in the present study, with feeding incidence increasing from 37.8 ± 4.26 % on 15 dph to 54.4 ± 4.26 % on 22 dph, while gut fullness increased from 20.03 ± 3.66 % on 15 dph to 24.9 ± 4.96 % on 22 dph. At the same time, we identified two periods of high mortality. The first high mortality period was registered shortly after the first introduction of feed (10-12 dph), which was probably driven by the challenging transition from endogenous to exogenous feeding processes. Interestingly, highest survival throughout the first feeding window and until 19-20 dph, was registered for larvae fed Diet 1. Moreover, at 15 dph, larvae fed Diet 1 were longer and larger, while their expression of genes related to digestion (*amyl2a*, *tgl*),

food intake (*pomca*), and growth (*gh*) was upregulated compared to the other Diets, indicating an initial benefit of this Diet for first-feeding European eel larvae.

However, the second period of high mortality occurred approximately around 20-24 dph, which is commonly described by fish larvae that do not successfully take up first-feeding or initiate feeding too late (or on the wrong diet). Here, despite the deceiving late feeding behaviour in presence of food items, the ability to search, capture, and assimilate nutrient fails, leading to irreversible mortality (Blaxter, 1974; Kamler, 1992). As such, the eel larvae in the present study probably reached the so-called "*point of no return*", which is further supported by the continuous upregulation (peaking at 22 dph) of *ghrl*, the gene encoding ghrelin, which is often referred to as the "*hunger hormone*", indicating that most eel larvae were fasting. Interestingly though, larvae fed Diet 3, downregulated the expression of *ghrl* again beyond 22 dph, probably demonstrating that those larvae did not fail in first feeding and thus, were not starving, resulting in survival beyond the "*point of no return*".

In addition to the improved survival, the bigger size (length and body area) and higher dry weight at 22 dph, in connection with the generally higher gut-fullness registered in larvae fed Diet 3, confirm that this diet has benefited eel larvae during this phase of the feeding window. Moreover, at the same time (22 dph), the upregulated expression patterns of the genes encoding the major digestive enzymes (*try*, *tgl*, and *amyl2a*) in larvae fed Diet 3, demonstrate that this benefit occurred also on the molecular level. Interestingly, for larvae fed Diet 1, expression of genes related to digestion (*try*, *tgl*, *amyl2a*), food intake (*pomca*), and growth (*gh*) showed a "dropping" pattern already at 22 dph (Fig. 8A), indicating the unsuitability of this diet in connection to a molecular decline in functionality of the associated mechanisms and processes. On the contrary, for larvae fed Diet 3, the expression patterns of genes related to digestion (*try*, *tgl*, *amyl2a*), food intake (*pomca*), and growth (*gh*) continued to increase towards 28 dph

(Fig. 8B), probably indicating the success of this diet and an improving maturity and functionality of the corresponding molecular feeding, digestion, and growth mechanisms.

In this regard, the success of Diet 3, can probably be partly attributed to the lower amount of lipids compared to Diet 1. This is further supported by the results of a previous study, where decreasing the lipid content from 40.4 % to 26.3 %, using a diet based on defatted shark eggs was shown to positively affect the survival and growth performance of Japanese eel larvae (Furuita et al., 2014). Similarly, adjusting the dietary lipid concentration from 10 to 30 % has been reported to enhance digestive tract maturation and improve larval development in European sea bass, *Dicentrarchus labrax* (Zambonino Infante and Cahu, 1999). As such, it seems that an appropriate amount of lipids in fish larval diets could be around 30 %, as also suggested for red drum, *Sciaenops ocellatus* (Buchet et al., 2000).

Furthermore, additionally to the main protein fraction, originating from dogfish egg yolk, the Diets 2 and 3 include an extra source of protein compared to Diet 1. Due to the high growth potential of fish larvae, the type of dietary proteins and their digestibility are key factors for development and survival of healthy organisms (Rønnestad et al., 2001; Zambonino Infante and Cahu, 2007). As such, proteins from fish hydrolysates (commonly di- and tripeptides, with molecular weights of ~3 kDa), are efficiently absorbed by the intestine and do not need to be pre-digested by the pancreas (Canada et al., 2017; Carvalho et al., 2003). Therefore, including hydrolysed proteins in fish larval diets has been demonstrated to positively affect survival and development of the digestive functionality in European sea bass, *Dicentratus labrax* (Zambonino Infante et al., 1997), survival of goldfish, *Carassius auratus* (Szlaminska et al., 1991) and growth of Atlantic salmon, *Salmo salar* fry (Berge and Storebakken, 1996). Consequently, we expected that the incorporation of protein hydrolysates in Diet 2 would improve growth and survival of eel larvae. However, eel larvae fed Diet 2 showed lower survival and

growth compared to larvae fed Diet 3. In this regard, too high inclusion of hydrolysed protein can have negative effects on larval performance as previously reported for Senegalese sole, *Solea senegalensis* (Canada et al., 2017), gilthead sea bream, *Sparus aurata* (de Vareilles et al., 2012; Kolkovski and Tandler, 2000), Asian sea bass, *Lates calcarife* (Srichanun et al., 2014) and Atlantic halibut, *Hippoglossus hippoglossus* (Kvåle et al., 2009, 2002). In those cases, the lower larval performance has been attributed to a saturation of the transport system in the intestinal brush-border membrane, due to overloading of short peptides (di- or tripeptides), causing an imbalanced utilization of free amino acids and resulting in a decreased protein accretion (Canada et al., 2019). As such, Diet 3, containing whey (molecular weight of approximately 10 kDa) as an extra source of protein, seems to be a more suitable diet for European eel larvae in the long term, especially beyond 22 dph. Here, despite the initial benefit of Diet 1 described above, supported by early morphological and molecular advantages, it seems that the inclusion of more complex dietary proteins is beneficial in order to maintain the European eel larval body size and develop throughout and beyond the first feeding window.

As such, Diet 3 seems to be able to sustain survival of European eel larvae, at least until the end of the present experiment (28 dph), where 4 % of larvae were still alive when fed Diet 3, compared to only 0.5 % of larvae fed Diet 2. In this regard, mortality during early life stages of fish in nature is expected to be high and averages 96.40 % and 99.98 % for freshwater and marine species, respectively (Houde, 2008). On the other hand, from an aquaculture perspective, the survival of 4 % seems low, however, when Japanese eel larval culture research was at a comparable stage, the survival rate of Japanese eel larvae showed a similar survival rate to this study, ranging between 3 to 5 % (Tanaka et al., 2001). Interestingly though, in the present study, the larvae fed the Japanese inspired Diet 1, did not survive long beyond the point of no return, probably indicating that either the recipe for this diet is still slightly different to the Japanese original

and/or that other feeding processes and techniques need to be improved. Nevertheless, the survival rate we observed in the present study, reaching 51.9 % at 20 dph, was an improvement compared to the survival rate (21.6 %) reported for European eel larvae fed previously tested diets in Benini et al., (subm., Study 3) at the corresponding comparable age. However, further research is still needed to improve current and identify new diet formulations and dietary regimes, facilitating growth and survival rate in European eel larval culture. Moreover, not only the quality of diets needs to be improved, but also the feeding procedures, tank design, and hygiene, as well as water quality.

To summarize, we observed an early benefit of Diet 1, supported by initially improved larval survival, morphology and gene expression, but it seems that the inclusion of more complex dietary proteins (as in Diet 3), but not hydrolysed peptides (as in Diet 2) is beneficial in order to promote larval ontogenetic performances after the successful transition to exogenous feeding. Here, European eel larvae fed with Diet 3 were able to survive throughout and beyond the first feeding stage, including longer-term morphological and molecular advantages compared to larvae fed Diet 1 and 2. As such, this indicates that the digestive and assimilation capacity of European eel larvae may vary throughout ontogeny and consequently, the dietary regime might need to be adapted according to stage specific nutritional requirements and preferences. However, further research is needed to improve larval feeding and rearing techniques, but the results of the present study provide an unprecedented step towards establishing a first feeding culture of hatchery produced European eel larvae and a basis for future research efforts towards sustainable aquaculture of this critically endangered species.

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Credit authorship

All authors contributed to this manuscript. **Elisa Benini**: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Kasun A. Bandara**: Methodology, Investigation, Writing – review & editing. **Sebastian Nikitas Politis**: Conceptualization, Methodology, Investigation, Data curation, Validation, Writing – review & editing, Supervision, Funding acquisition. **Sofia Engrola**: Conceptualization, Methodology, Data curation, Validation, Writing – review & editing, Supervision. **Anders Nielsen**: Formal analysis, Data curation, Validation, Writing – review & editing. **Luis E.C. Conceição**: Conceptualization, Methodology, Resources, Data curation, Writing – review & editing. **Jonna Tomkiewicz**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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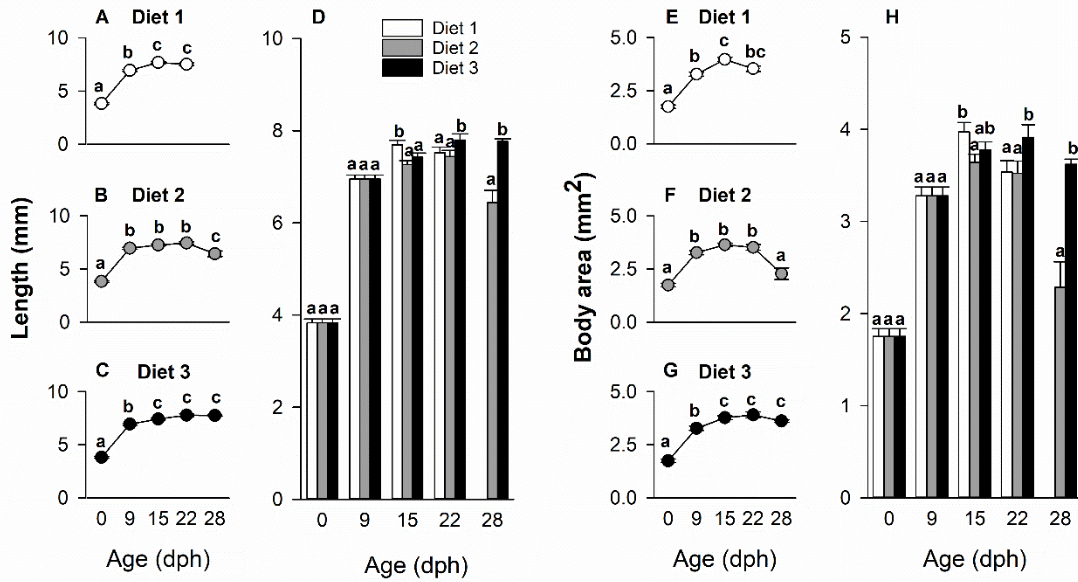

Figure 15 - Length and body area in relation to age for larvae of European eel, *Anguilla anguilla*, fed three experimental diets (1, 2 and 3). Effect of age for each diet on standard length (A-C) and body area (E-G) and effect of diets at each age for standard length (D) and body area (H). Assessment occurred at specific developmental points such as hatch (0 dph), before first feeding (9 dph), during first feeding (15 dph), end of the first feeding period (22 dph) and beyond the first feeding period (28 dph). Values represent means (± SEM), while different lower case letters represent significant differences ($p < 0.05$).

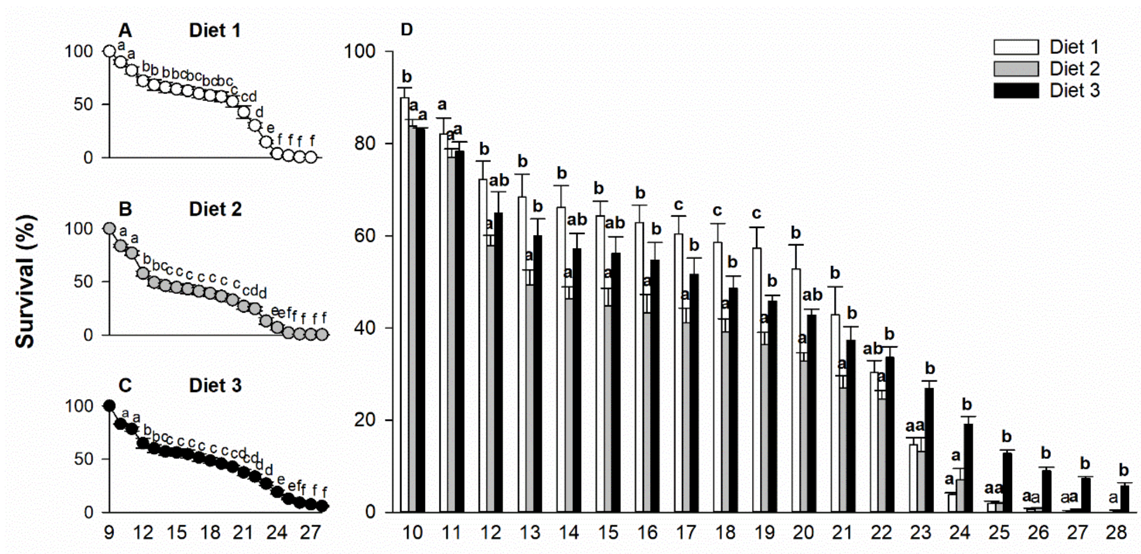

Figure 2 - Effect of age for each diet (A, B, C) and effect of diets at each age (D) on European eel*, Anguilla anguilla* larval survival. Values represent means (± SEM), while different lower case letters represent significant differences ($p < 0.05$).

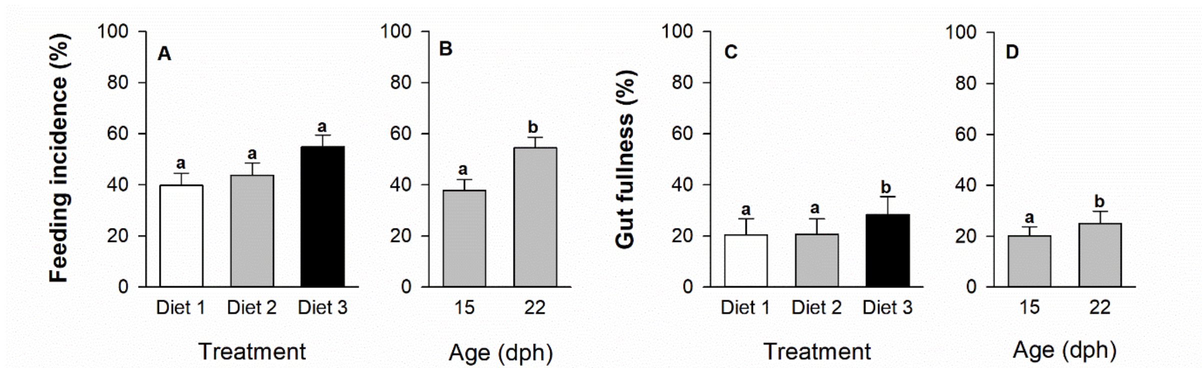

Figure 3 - Effect of age and treatment (diet) on larval European eel, *Anguilla anguilla* feeding incidence (%) and gut fullness (%). Values represent means (± SEM), while different lower case letters represent significant differences ($p < 0.05$).

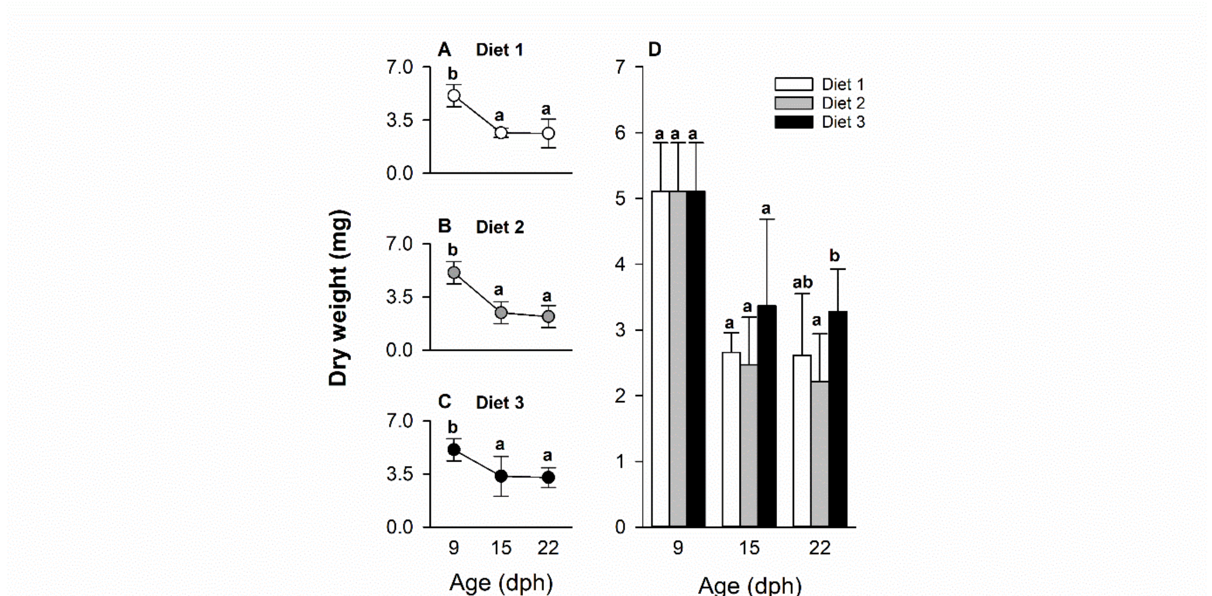

Figure 4 - European eel, *Anguilla anguilla* dry weight (mg/ind) during larval development. Effect of age at each diet (A-C) and effect of diets at each age (D). Values represent means (± SEM), while different lower case letters represent significant differences ($p < 0.05$)

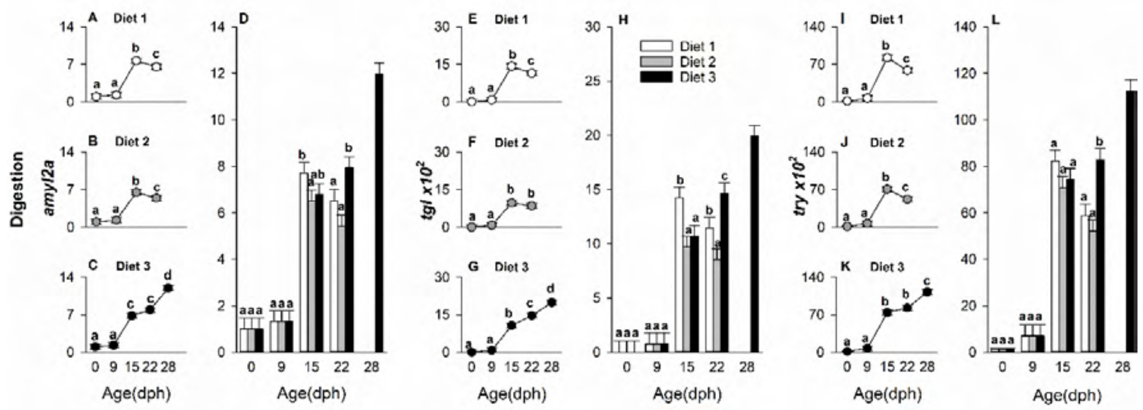

Figure 5 - Relative expression of amylase (*amyl2a*), triglyceride lipase (*tgl*) and trypsin (*try*) in European eel, *Anguilla anguilla* larvae. Effect of treatment (diet) at each age (A-C, E-G, I-K) and effect of age (dph) at each treatment (D, H, L). Values represent means (± SEM), while different lower case letters represent significant differences ($p < 0.05$).

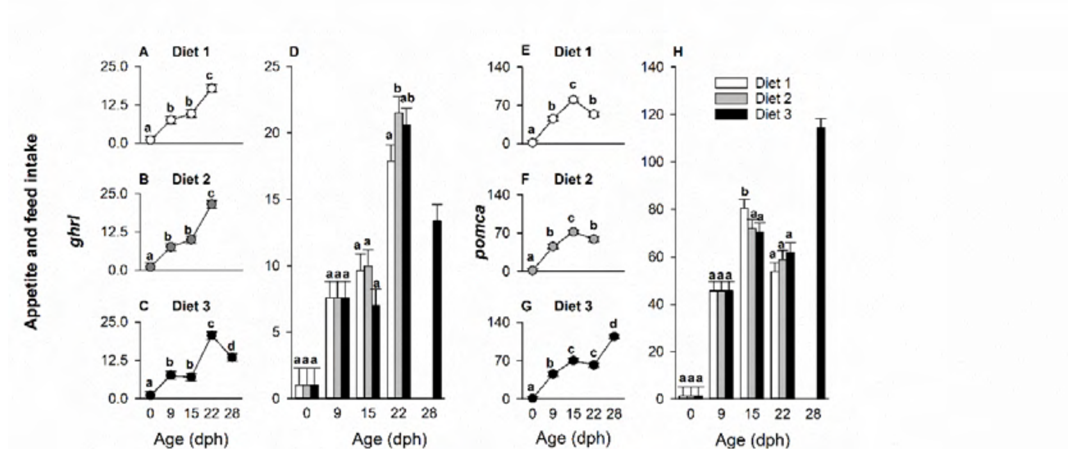

Figure 6 - Effect of age (dph) and treatment (diet) on relative expression of ghrelin (*ghlr*) and proopiomelanocortin (*pomca*) in European eel, *Anguilla anguilla* larvae. Values represent means (± SEM), while different lower case letters represent significant differences ($p < 0.05$).

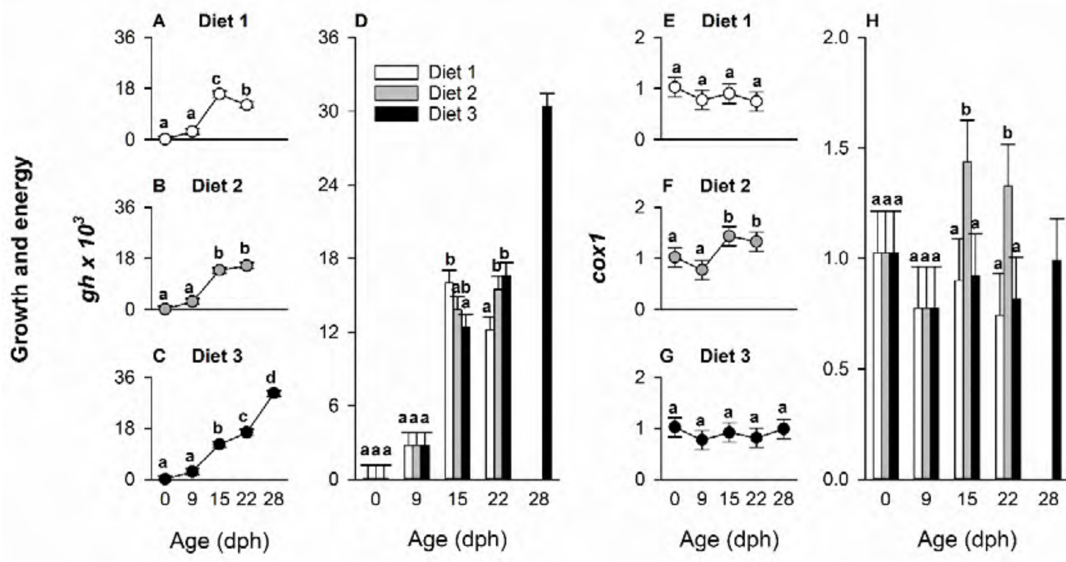

Figure 7 - Effect of age (dph) and treatment (diet) on relative expression of growth hormone (*gh*) and cytochrome c oxidase (*cox1*) in European eel, Anguilla anguilla larvae. Values represent means (± SEM), while different lower case letters represent significant differences ($p < 0.05$).

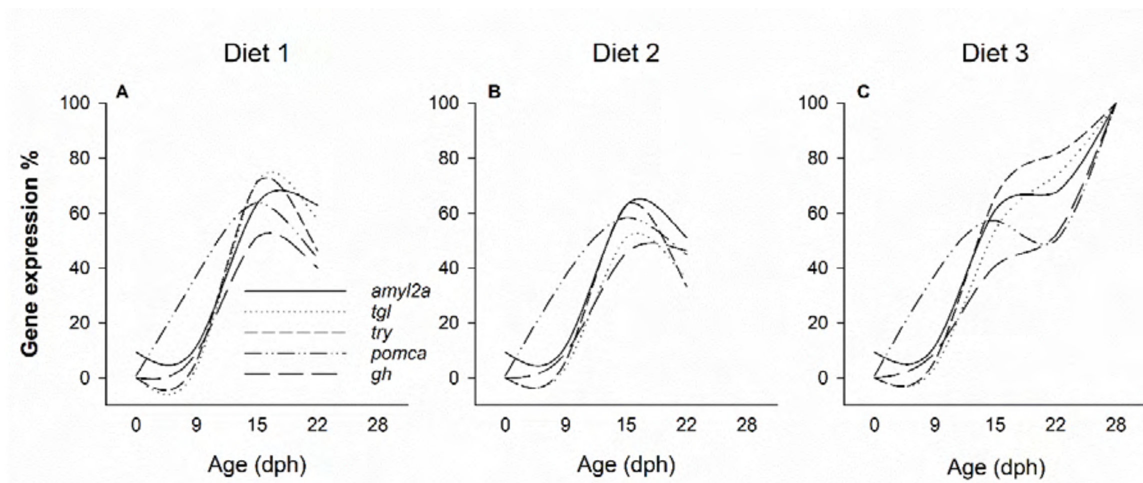

Figure 8 - Overview, showcasing the difference in relative expression of selected genes (*try, amyl.2a, tgl, pomca* and *gh*) in European eel, *Anguilla anguilla* larvae fed Diet 1 (A), Die 2 (B) and Diet 3 (C) throughout the experimental period (from 0 to 28 dph).

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