European eel larval ontogeny and physiology Insights at morphological and molecular level

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PhD Thesis

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PhD thesis

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2018

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EEL-HATCH

Contents

Preface

This thesis, submitted in partial fulfillment of the requirements for the Doctor of Philosophy Degree (PhD), was performed at the Technical University of Denmark, National Institute of Aquatic Resources (DTU Aqua) within the Fish Biology Research Group of the Section for Marine Living Resources.

Moreover, this PhD fellowship was part of the research and innovation project: *Eel Hatchery Technology for a Sustainable Aquaculture* (EEL-HATCH) coordinated by DTU Aqua and supported financially by Innovation Fund Denmark, Grant no.5184-00093B. As such, the research presented in this thesis was conducted between 2014 and 2017 at two research facilities of DTU Aqua as an integral part of the EEL-HATCH project, under the supervision of Jonna Tomkiewicz and Ian A.E. Butts. The first experimental series were carried out at Lyksvad Fish Farm in southern Jutland, while the later series took place at the eel hatchery built as part of the EEL-HATCH project in Hirtshals, northern Jutland. Here, the integration into a multidisciplinary team of researchers and industrial partners has provided the opportunity to conduct comprehensive experiments with European eel offspring, at the cutting edge of research towards the development of larval culture for this extraordinary species.

The studies of the thesis focus on the ontogeny and physiology of the early larval stages, ranging from hatch to first-feeding, with emphasis on biotic and abiotic factors influencing early life history. Here, sampled larvae from dedicated experiments related to temperature, salinity and first-feeding, were the source for extensive morphological and molecular analyses. These molecular analyses supporting records on development and survival were supervised by José-Luis Zambonino-Infante, David Mazurais and Arianna Servilli at the French Research Institute for Exploitation of the Sea (IFREMER), in Plouzane, France and by Joanna Miest and Catriona Clemmesen at the Helmholtz Centre for Ocean Research (GEOMAR), in Kiel, Germany.

It has been invaluable to be part of an international team with leading expertize in European eel research, also prior to initiating my PhD, where I gained invaluable hands on experience. It was here, the research in larval culture became an opportunity as the enhanced assisted methodology developed in the EU-project: *Reproduction of European Eel: Towards a Self-sustained Aquaculture* (PRO-EEL) enabled a stable production of viable eggs and regular mass hatch of larvae. This opened an array of unanswered questions about physiological requirements and rearing conditions of the unpreceded culture of European eel larvae, complicated by the lack of knowledge regarding the early life history stages in nature.

The aim of this PhD was in this context to fill gaps in knowledge about requirements of early life history stages, enhancing survival and leading to first-feeding stages during larviculture of European eel. Altogether, this PhD project has provided a unique opportunity to enhance my skills in designing, performing and analyzing experimental studies as well as learning the methodology, application and interpretation of molecular tools. In conclusion, I am proud to say that my research has contributed to the increased survival through the yolksac stage, which now allows hundred thousands of European eel larvae to enter the first-feeding stage and feeding experiments.

English summary

The research conducted within this PhD project contributes to filling gaps in knowledge about the enigmatic life cycle of European eel (*Anguilla anguilla*) by addressing biotic and abiotic factors influencing early larval stages. This involves experimental studies and utilization of *state-of-the-art* molecular tools elucidating links between morphology and molecular mechanisms in the quest to identify suitable rearing and feeding conditions for larviculture. As such, this thesis comprises six studies within three main topics: i) temperature, ii) salinity and iii) nutrition, influencing larval development and survival.

The first three studies address the influence of temperature on early larval ontogeny. Here, **Study 1** determined the thermal tolerance limits and identified an intermediate thermal environment for future larval culture with efficient growth and low frequency of deformities associated with high expression of growth hormone (improved growth) and low expression of heat shock proteins (decreased stress). Moreover, **Study 2** revealed that expression of genes encoding thyroid hormone receptors and deiodinases, associated with the mediation of thyroid hormone action, show sensitivity to temperature and are involved in and during early larval development. Additionally, **Study 3** shed light on the molecular ontogeny of the larval immune system under different thermal scenarios and identified an immune-compromised phase during which mortality is high and larvae are more vulnerable to pathogen infection. This will have important implications on rearing conditions and disease prevention protocols in eel hatcheries but also improve our understanding of ocean warming impacts on fish recruitment. Thereafter, experimental work focused on the salinity tolerance of these marine larvae. Here, **Study 4** clearly demonstrated that culture regimes reducing salinity towards iso-osmotic conditions facilitated enhanced European eel pre-leptocephalus development and survival revealing the existence of underlying, highly sensitive and regulated osmoregulation processes in the early larval stage. This novel insight gained by morphologically and molecularly defined physiological thermal and osmoregulatory tolerance limits and preferences gradually led to improved protocols for pre-leptocephalus larval culture and resulted in significantly increased numbers of healthier and stronger larvae reaching the first-feeding stages. Previous to these culture condition improvements, a pilot nutritional trial was performed. Here, **Study 5** for the first time explored several diets, tested attractants and described behavioral feeding patterns of European eel larvae. Finally, with much enhanced numbers of larvae and using the previously identified benchmark diet as well as enhanced temperature condition, **Study 6** revealed that initiation of exogenous feeding in European eel occurs concurrently with the onset of the genetically pre-programmed underlying hormonal control of ingestion and the enzymatic regulation of digestion, known to regulate physiological functions of feeding. The here gained knowledge, improved the understanding of an undisclosed phase of the European eel life cycle, which is the transition from yolk-sac pre-leptocephalus larvae to the exogenous feeding leptocephalus stage and constitutes essential information in order to develop efficient feeding strategies for future larviculture of this species.

In conclusion, the conducted research elucidated molecular aspects of important biological processes in order to more closely understand the complexity of regulations involved in early European eel ontogeny and physiology. The gained knowledge contributes to our understanding of unknown mysterious aspects of the European eel life cycle and most importantly provides promising steps for eel aquaculture towards completing the life cycle in captivity of this socially and economically important as well as critically endangered fish species.

Dansk resumé

Forskningen gennemført i dette PhD-projekt bidrager til at udfylde huller i vores viden om den europæiske åls gådefulde livscyklus ved at adressere biotiske og abiotiske faktorer, der påvirker de tidlige larvestadier. Den ny viden er opnået gennem kontrollerede forsøg og anvendelsen af moderne molekylære metoder til at belyse sammenhængen mellem den morfologiske udvikling og de underliggende mekanismer med henblik på at identificere egnede opdrætsforhold i larvekultur. Overordnet omfatter afhandlingen seks studier inden for tre hovedemner, nemlig indflydelsen af: i) temperatur, ii) saltholdighed og iii) ernæring på larvernes udvikling og overlevelse.

De første tre studier omhandler temperaturens indflydelse i de tidligste larvestadier. Det indledende studium, **Studie 1**, som fokuserede på larvernes tolerancegrænser over for vandtemperaturen, identificerede et temperaturinterval et par grader lavere end det normalt praktiserede som værende det mest gunstige for larvekultur. Kendetegnene var en mere effektiv vækst og lavere frekvens af deformiteter forbundet med høj ekspression af væksthormon (forbedret vækst) og lav ekspression af "heat-shock" proteiner (nedsat stress). Sammenfaldende viste **Studie 2** relateret til skjoldbruskkirtlens funktion herunder stofskifte, at ekspressionen af gener, som koder for hormonreceptorer og deiodinaser, er temperaturfølsom, og at begge dele er involveret i den tidlige larveudvikling. Resultaterne blev yderligere understøttet af **Studie 3**, som identificerede en temperatursensitiv fase i immunsystemets udvikling, hvor larverne er særligt sårbare over for sygdomme og dødeligheden derfor høj. Temperaturregimet kan her vise sig at have stor betydning for sygdomsforebyggelse og larveoverlevelse i klækkerier og desuden øge forståelsen af, hvordan øgede havtemperaturer kan påvirke fiskelarvers overlevelse i naturen. Det følgende eksperiment, **Studie 4**, fokuserede på tolerancen overfor vandets saltholdighed hos disse marine fiskelarver. Her viste resultaterne, at en reduktion af saltholdigheden mod iso-osmotiske tilstande, øgede overlevelsen og nedsatte frekvensen af deformiteter hos larverne, og afslørede underliggende, følsomme og fint regulerede osmoreguleringsprocesser i det tidlige larvestadium. Den nye indsigt opnået gennem både morfologisk og molekylært definerede fysiologiske tolerancegrænser samt præferencer for temperatur og saltholdighed resulterede i forbedrede protokoller til kultur af blommesæklarver. Disse resultater har bidraget til en signifikant forøgelse af antallet af sunde, stærke larver, der når til det første fødesøgende stadium. Allerede tidligt i arbejdet med forbedringer af kulturforholdene blev der udført pilotforsøg med fodring af larver. Således gennemførtes i **Studie 5** de første dokumenterede forsøg med fodring af larver af europæisk ål og deres fourageringsadfærd blev dokumenteret for første gang. Senere i **Studie 6** blev flere fodringsforsøg gennemført med et højere antal larver på baggrund af det forbedrede temperaturregime og øget vandkvalitet. Her anvendtes den bedste diæt fra pilotstudiet. Forsøget identificerede tidspunktet for overgangen fra endogen til eksogen fødeoptagelse hos ålelarverne, hvor også den hormonelle kontrol af fødeindtagelse og den enzymatiske regulering af fordøjelsen kunne påvises at indtræde. De her opnåede resultater har skabt indsigt i en hidtil ukendt fase af den europæiske åls livscyklus, nemlig overgangen fra blommesæklarve til bladlarve, hvilket er dér, hvor larverne begynder aktivt at tage føde til sig. Disse resultater vil være væsentlige for udviklingen af foder og fodringsstrategier for fremtidens larvekultur af den europæiske ål.

Tilsammen belyser den gennemførte forskning indflydelsen af biotiske og abiotiske forhold på ålelarvernes overlevelse og udvikling, inklusiv molekylære aspekter af vigtige biologiske processer, hvilket bidrager til at forstå kompleksiteten i de tidlige larvestadiers ontogeni og fysiologi. Den opnåede viden bidrager til vores forståelse af ukendte dele af den europæiske åls livscyklus og er et lovende skridt i retning af at lukke livscyklus i akvakultur for denne økonomisk og kulturet værdifulde fisk og samtidig kritisk truede art.

1. European eel

1.1. Life cycle

The mysterious life cycle of the diadromous European eel, *Anguilla anguilla*, includes oceanic and continental phases, but is in its complexity still not fully understood (***Fig. 1***). It is believed that spawning takes place in the Sargasso Sea area (Schmidt, 1923), which is supported by the presence of relative newly hatched larvae but not by actual spawning adults. Once in the Sargasso Sea, eels are assumed to spawn in late winter and spring. Adult eels are further on assumed not to leave the Sargasso Sea after spawning, while their leaf-shaped offspring called "leptocephali" use prevailing currents on their migration back to the continental shelf of Europe and North Africa. This journey takes approximately 200-300 days. Before entering coastal zones, estuaries and rivers, the larvae metamorphose into the transparent juvenile stage called "glass eels". As they further on move into freshwater habitats, they gain pigmentation and transform into so called "elvers" and then "yellow eels" (Tesch, 2003).

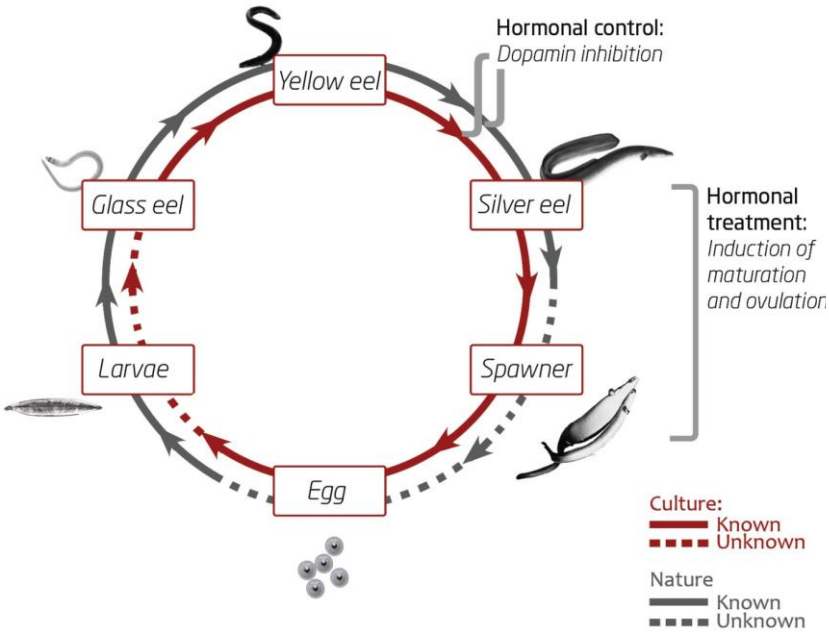

Figure 1: European eel life cycle with known and unknown parts in nature and culture. Adopted from www.pro-eel.eu

Age-at-maturity varies according to latitude, ecosystem characteristics, and density-dependent processes. Males reside in freshwater habitats for a period of 6-12 years while females linger for 9-18 years. At a still uncertain point of their freshwater life, eels undergo sexual maturation (silvering). During that period, their eyes become bigger, their heads broader and the content of body fat increases before they turn agastric (cease feeding) on their migratory journey towards their oceanic spawning areas in the Sargasso Sea (FAO 2018).

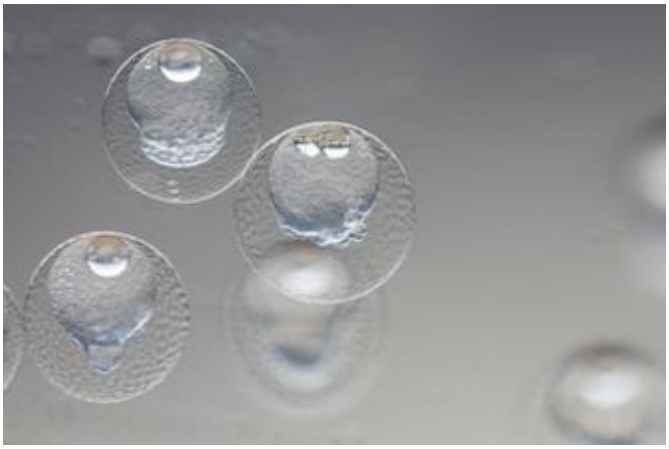

Figure 2: European eel eggs have never been encountered in nature and are thus only known from *in-vitro* experiments. Photo: Sune R. Sørensen

1.2. Spawning habitat

Regarding the exact spawning location for European eel, some question marks still remain, as the earliest life stages of this species have still not been encountered in nature (***Fig. 2***). However, based on the spatio-temporal distribution of larvae of different sizes, Johannes Schmidt was able to delimit their spawning location to the western Atlantic Ocean (Sargasso Sea), which was further confirmed by the so far smallest European eel pre-leptocephalus larvae captured at depths between 50 and 350 m (Castonguay and McCleave, 1987; Schoth and Tesch, 1984).

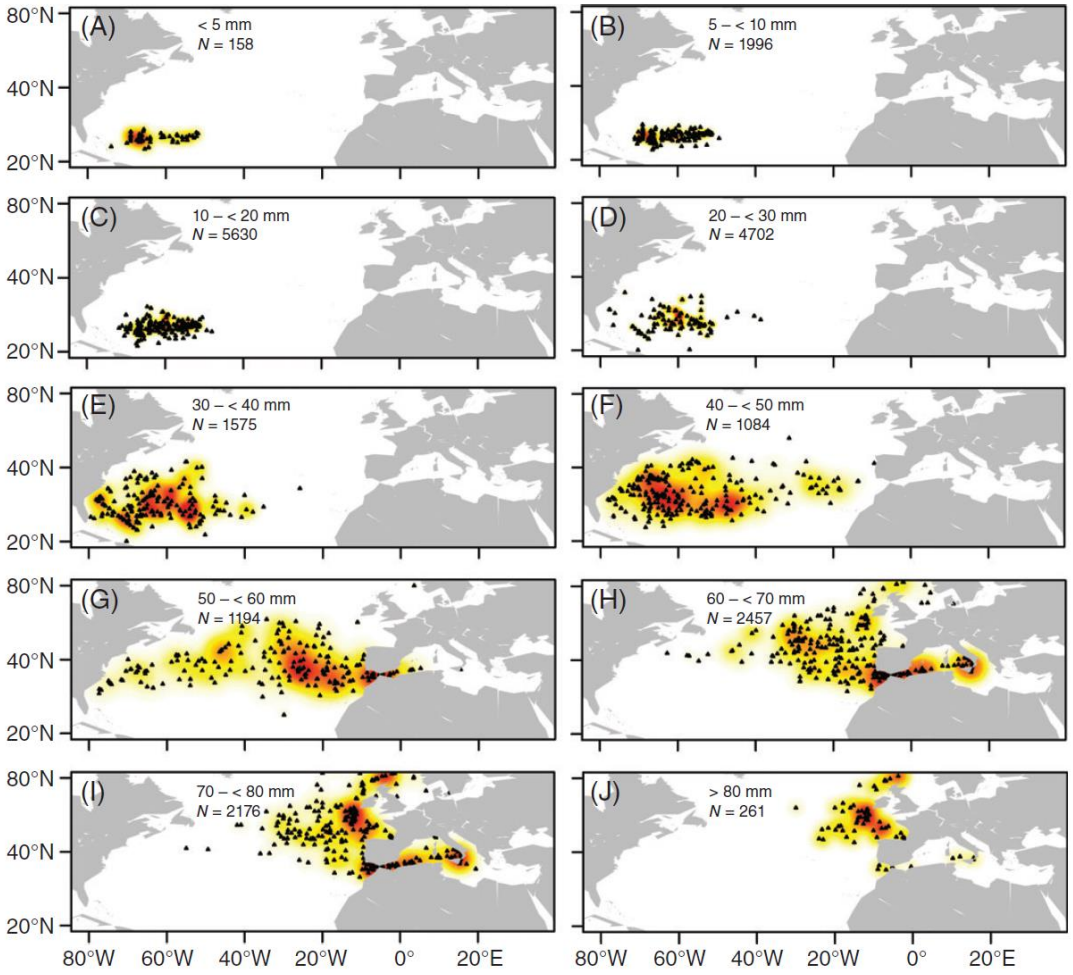

Figure 3: Collection locations of different size classes of *Anguilla anguilla* leptocephali (A–J) in the North Atlantic and Mediterranean Sea (black triangles). The spatial density of the number of larvae caught in each area is represented by yellow and orange (highest density) contour shading. Adopted from Miller et al., 2015.

Slightly older European eel leptocephalus larvae are found to be present in the upper 100 m layer at night and migrate to greater depths with lower light intensity during daytime (Castonguay and McCleave, 1987; Schoth and Tesch, 1984). Deeper distributions of later developmental stages (60-85 mm) of European eel leptocephali have also been reported, i.e. at depths of 300-650 m during day (Tesch, 1980). Summarizing the past oceanic larval eel research, a schematic of the spatio-size distribution of European eel offspring encountered in the Atlantic Ocean (***Fig. 3***), demonstrates the eastward spreading as validated by the progressive size distribution (Miller et al., 2015).

1.3. Natural recruitment

Today, the European eel stock is considered outside of safe biological limits and as current fisheries are unsustainable, they are still listed as "critically endangered" by the IUCN Red List (Jacoby and Gollock, 2014). Additionally, the diminished natural stock leads to a reduced reproductive potential. Unfortunately, there has been a continuous decline of eel recruitment since the 1950s and marked decadal reductions in glass eel recruitment since the early 1980s. The annual recruitment of glass eel to European waters in 2017 remained low, at 1.6% of the 1960–1979 level in the "North Sea" series and 8.7% in the "Elsewhere Europe" series, while the annual recruitment of young yellow eel to European waters was 24% of the 1960–1979 level (***Fig. 4***). As those recruitment indices are still well below the 1960–1979 reference levels, there is no change in the perception of the stock status (ICES, WGEEL 2017). On top of that, they are fished throughout all life stages, including glass eels, yellow eels and migrating silver eels, which makes them particularly vulnerable considering that they reproduce only once during their lifetime.

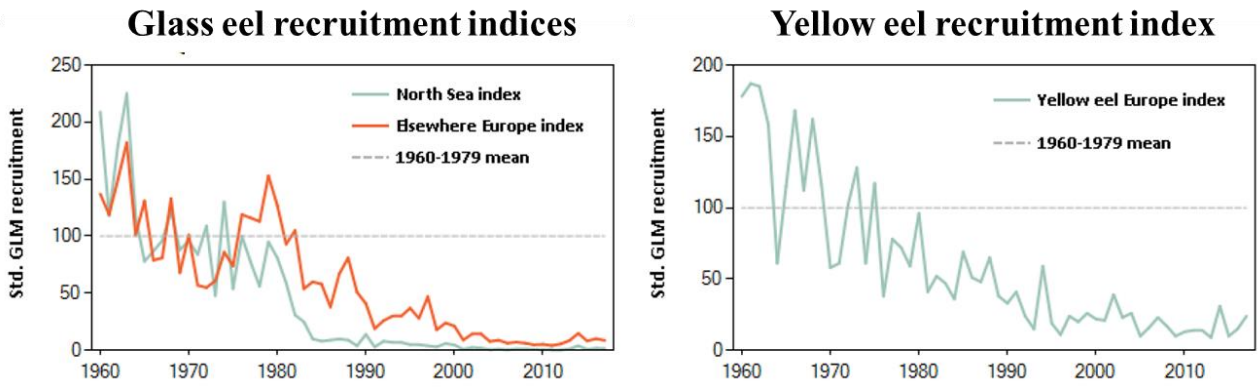

Figure 4: Left panel: Geomatric mean of estimated (GLM) European glass eel recruitment for the continental "North Sea" and "Elsewhere Europe" series. The "North Sea" series comprises data from Norway, Sweden, Germany, Denmark, the Netherlands, and Belgium. The "Elsewhere" series comprises data from UK, Ireland, France, Spain, Portugal, and Italy. Right panel: GLM of European yellow eel recruitment trends. Adopted from ICES WGEEL 2017.

Several causes are believed to have contributed to the decline of the eel stock including anthropogenic stressors such as overfishing, pollution, habitat degradation, connectivity barriers (e.g. hydropower plants), man-introduced parasites and diseases, as well as environmental factors such as ongoing oceanic and climatic changes (Knights 2003, Friedland et al., 2007; Bonhommeau et al., 2008; Van den Thillart et al 2009; Gutiérrez-Estrada and Pulido-Calvo 2015).

Additionally, as all eel stocks are declining, but the market demand is constantly increasing, an illegal European glass eel fishery has evolved to "feed" the thriving illegal trade against the ongoing export ban. Unfortunately, this "eel-mafia" has a turnover of more than 400 million Euro by smuggling glass eels, which is comparably profitable to illegal gun and drug trafficking (Arapovic and Pfeil, 2017). Probably, this is one of the most serious pressures driving the European eel decline.

1.4. Aquaculture

European eel is a targeted, high-value species for aquaculture, but eel farming is still a capture-based industry that relies totally on wild-caught glass eels, which are transferred into on-growing facilities and fed until reaching marketable sizes. Unfortunately though, as mentioned above, the natural populations and their reproductive potential have declined to a historical minimum, mainly due to climatic and anthropogenic pressures during the different phases of the eel life cycle (ICES, WGEEL 2017). Thus, similar to the glass eel recruitment pattern, the commercial glass eel fishery landings due to both lower stock abundance but also fishing regulations have declined to a diminishing level (***Fig. 5***), which subsequently led to the collapse of the European eel aquaculture industry, reaching a level of almost no commercial importance.

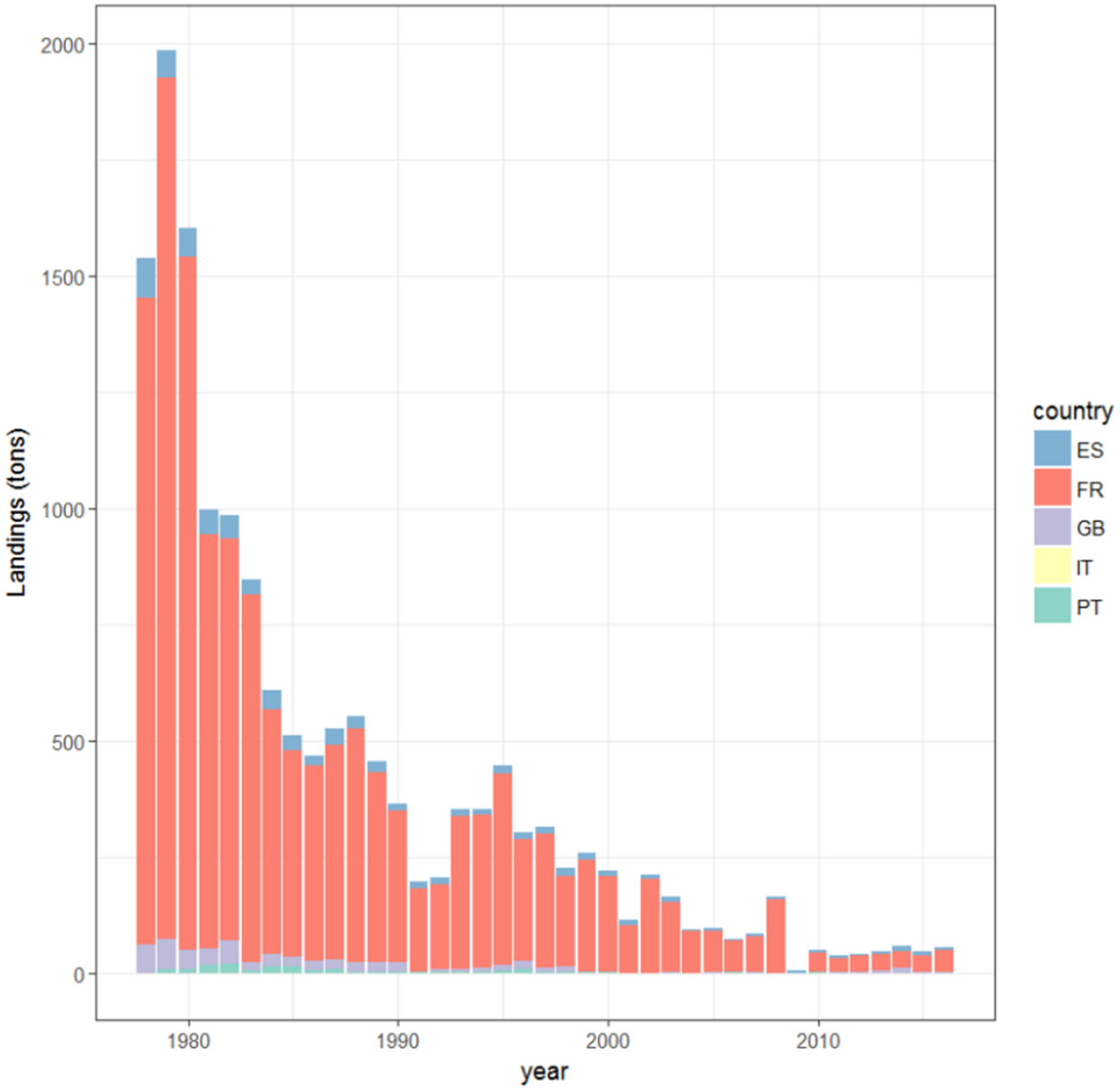

Figure 5: Commercial European glass eel fishery landings by country (reported). Adopted from ICES WGEEL 2017.

Consequently, European eel aquaculture production, which actually increased until the end of the 1990s, clearly started to decline since the mid 2000s from 8000-9000 tons to approximately 5000-6000 tons today (***Fig. 6***). In this context, the present critically low stock abundance of European eels, challenges the sustainability of the eel aquaculture industry and especially the associated unsustainable legal and in particular illegal fishing processes, urging the need to further develop and establish captive breeding techniques for this species. As such, advanced research towards assisted reproduction and subsequent early life history rearing conditions, is on-going to provide consecutive promising steps towards a sustainable aquaculture of this commercially important and critically endangered fish species.

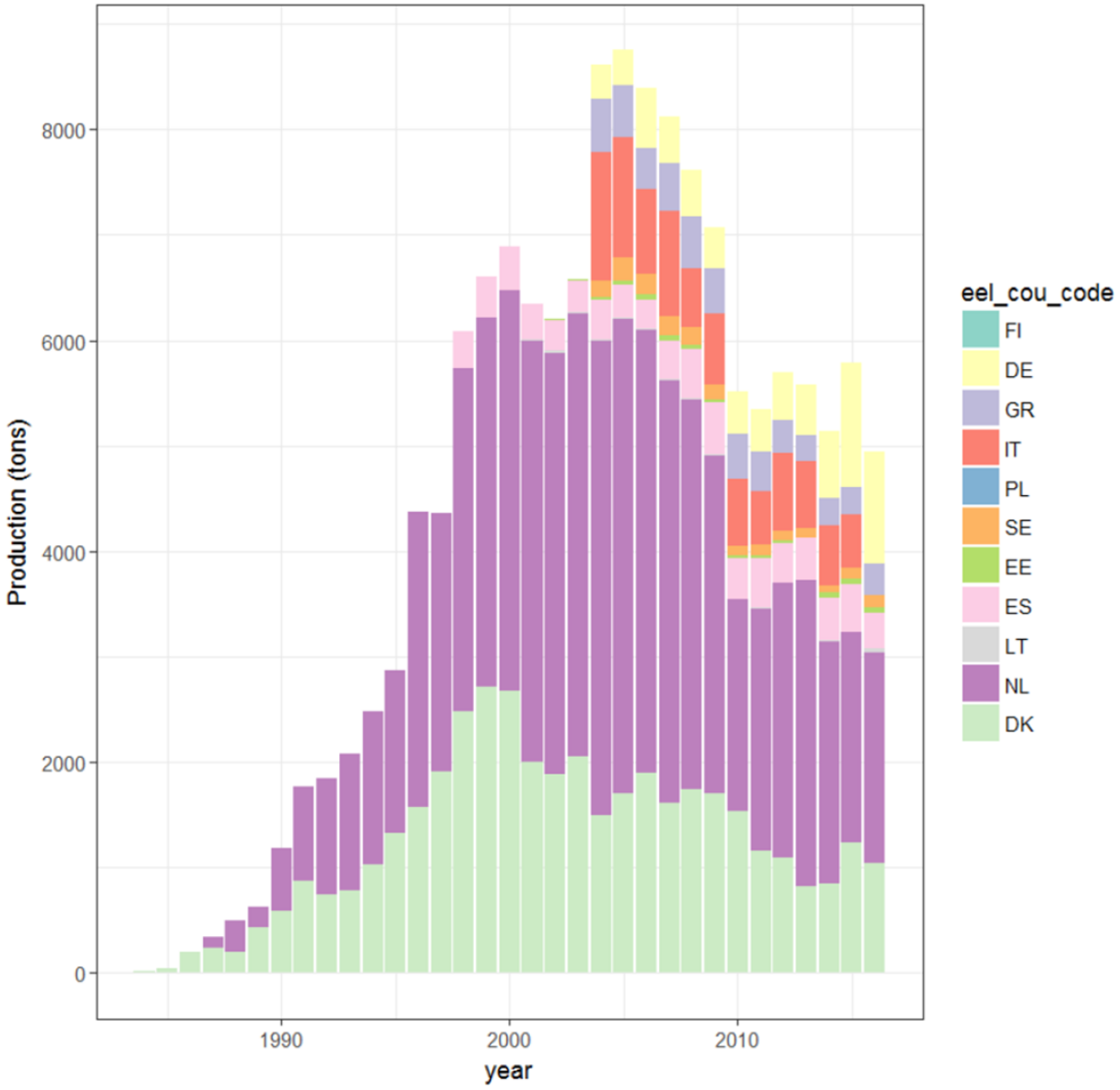

Figure 6: European eel aquaculture production (reported) by country since 1984. Adopted from ICES WGEEL 2017.

1.5. Progress in breeding

Eels do not reproduce naturally in captivity due to complex hormonal control mechanisms that relate to their long migration patterns to native oceanic spawning areas (Vidal et al., 2004). Such maturational barriers can be overcome through hormonally assisted reproduction, which led to the first report of European eel offspring obtained from artificially matured fish, more than 30 years ago (Bezdenezhnykh et al., 1983). Since then, eel research has gained scientific inquiry, especially regarding assisted reproduction, with similar results being reported almost two decades later (Pedersen et al., 2004; Palstra et al., 2005), though with still limited success.

Starting in 2010, the international and multidisciplinary research project "*Reproduction of European eel: towards a self-sustained aquaculture*" (PRO-EEL), coordinated by DTU Aqua and financially supported by the European Commission's 7th Framework Program under Theme 2 "*Food, Agriculture and Fisheries, and Biotechnology*", developed standardized protocols for captive European eel reproduction and thus moved the field from individual efforts of reproductive failure towards stable production of healthy gametes and viable offspring (Tomkiewicz, 2012; www.pro-eel.eu). Within this framework, standardized assisted reproductive techniques were developed including hormonal induction of gametogenesis, gamete handling procedures, sperm to egg ratios and *in vitro* fertilization processes, following stripped spawning methodologies (Sørensen et al., 2013; Butts et al., 2014), which in combination led to high fertilization success.

Figure 7: European eel hatchery built *via* the EEL-HATCH project in Hirtshals, northern Jutland, Denmark.

Additionally, first protocols were defined for embryonic and larval culture, where biophysical factors such as light regimes (Politis et al., 2014), microbial control (Sørensen et al., 2014), salinity/salt composition (Sørensen et al., 2016a) as well as systems and techniques for embryo incubation and larval rearing were addressed. This led to the first description of the early ontogeny of European eel (Sørensen et al., 2016b), however, with negligible survival to the feeding stage.

The research has continued in the Danish innovation project "*Eel Hatchery Technology for a Sustainable Aquaculture*" (EEL-HATCH; www.eel-hatch.dk). Here the European eel breeding technology has taken a further promising step through the establishment of a top modern eel hatchery (***Fig. 7***), which was built as part of the EEL-HATCH project, forming the basis for promising next steps especially regarding larval culture of this species.

1.6. Early life history

Similarly to all species new to aquaculture, the main obstacles during early life history that needed to be addressed were to explore suitable rearing conditions but also to identify potential feeding regimes. The choice of both, rearing conditions and potential food items used in aquaculture for a particular species is commonly based on the ambient conditions and food availability in their natural environment or based on experimental findings. However, in the case of European eel, the natural environmental regimes of embryos and earliest (pre-leptocephalus) larval stages remain unclear, as they have not been encountered in nature. Thus, research needed to focus on captive produced offspring and laboratory studies to overcome current bottlenecks and gain knowledge about the species early life biology.

The development of artificially produced eggs and embryos of European eel at a temperature of 20°C and salinity of 36 psu (which was considered state-of-the-art at initiation of this PhD project), has now been described by Sørensen et al., 2016b and is illustrated in ***Fig. 8***. Here, it is clear that the spherical positive buoyant eggs contain a characteristic large amount of smaller oil vesicles that slowly fuse together to form a large oil droplet, while the chorion separates from the plasma membrane to create the perivitelline space around the cytoplasm (***Fig. 8A-E***). At ~1 hour post fertilization (hpf) it is possible to observe the first cell cleavages (***Fig. 8C***), while the 16-cell stage is reached within ~4 hpf (***Fig. 8E***). After the blastula stage follows the formation of a germring at ~13 hpf, marking the onset of gastrulation (***Fig. 8G***). After ~15 hpf, the gastrula comprised ~50% of the yolk, i.e. 1/2 epiboly (***Fig. 8H***), while first somites became visible at ~24 hpf (***Fig. 8I***). At ~32 hpf, the embryo evolves two noticeable eye capsules and several somites (***Fig. 8J***). Thereafter, the tail bud is forming, the yolk sac becomes ellipsoid and hatching occurs at ~48 hpf (***Fig. 8L***).

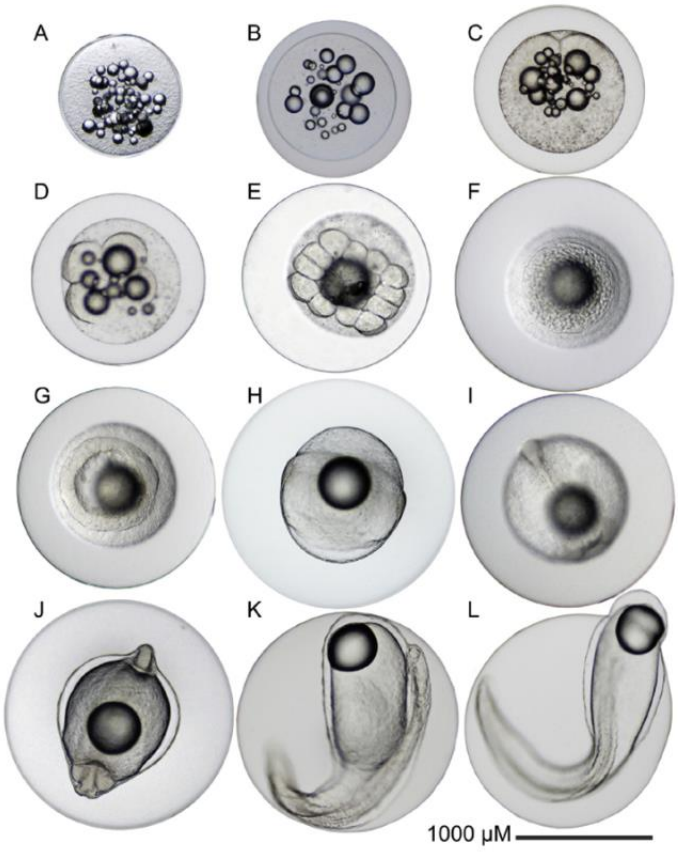

Figure 8: Embryonic development of European eel at 20°C. Adopted from Sørensen et al., 2016b.

Correspondingly, larval development throughout the yolk sac stage, at the same conditions as previously mentioned, is illustrated in ***Fig. 9***. At hatch, European eel larvae seem relatively undeveloped with a rather prominent large yolk-sac and oil droplet, enabling the positive larval buoyancy (***Fig. 9A***). Thereafter, larvae utilize their energy reserves to grow in length and develop optical capsules, a visible hindbrain and pericardium as well as a wide primordial fin with a well-defined, rounded and pigmented tail (***Fig. 9B***). Moreover, the oral opening was first visually evident as a small channel posterior to the eyes and underwent pronounced changes towards 5 dph (***Fig. 9C***). At ~8 dph, the eyes started to pigment, the upper and lower jaws developed and early stages of teeth appeared (***Fig. 9D***). The angle between the head and trunk increased to enable the upper and lower jaws pointing forward, finally resulting in the formation of the feeding apparatus of the first-feeding European eel larvae with the characteristic, protruding teeth. All yolk reserves were gone at ~14 dph with no evident morphological change beyond this point (***Fig. 9E***).

Figure 9: European eel pre-leptocephalus larval development at 20°C from hatch until 14 days post hatch (dph). Modified from Sørensen et al., 2016b.

Thus, a gap in knowledge regarding the European eel embryonic and larval stages (still undiscovered in nature) has been filled (Sørensen et al., 2016b), providing the basis for the larval research and technology development within EEL-HATCH and thus also this PhD project. Here, the goal was to identify rearing conditions optimizing larval survival and welfare with emphasis on biotic and abiotic factors influencing early life history but also to explore potential first-feeding regimes. These studies have naturally been inspired by insights regarding the ontogeny and physiology of Japanese eel early larval stages (e.g. Ahn et al., 2012; Okamura et al., 2016), as research and hatchery technology development for this species is by far more advanced (Tanaka, 2015) and even supplemented by new insights gained from nature as well as the identification of their natural habitat (e.g. Tsukamoto et al., 2011). As the present work is pioneering in its field, detailed morphological analyses and molecular tools were applied to gain *state-of-the-art* insight.

2. Molecular tools

This PhD project utilized the advancement and availability of molecular tools to complement morphological findings in the quest to identify suitable rearing and feeding conditions during European eel early life history rearing. As such, the here conducted research elucidated aspects of processes taking place during eel larval culture in order to more closely understand the complexity of regulations involved in early ontogenesis.

Today, rapidly advancing, increasingly accessible and economically affordable molecular methods and tools are revolutionizing research, especially when genetics intersect other life sciences. Thus, it is increasingly common to study genetic processes such as gene structure and function as well as variation or distribution but also what is referred to as "nature *vs.* nurture", where an organismal development and behavior is intrinsically-genetically pre-programmed and/or influenced by extrinsic factors (Moore, 2003).

The increasing feasibility to sequence complete genomes has facilitated an increasing amount of genomic studies providing unprecedented insights for some model species, which further enabled investigations on several more non-model species. Over the last decade, increased scientific inquiry has focused on developing and applying genomic tools to better understand underlying mechanisms of physiology during ontogenetic development in fishes and to explore the impact of environmental or nutritional factors even during the most delicate periods in early life history (reviewed in Mazurais et al., 2011). In the case of the critically endangered European eel, the genome was recently sequenced and assembled (Henkel et al., 2012; Jansen et al., 2017), offering new perspectives for eel research in order to identify actors involved in different biological processes, cellular components and molecular functions that are of importance in this species.

It is widely accepted that regulation of gene expression is a fundamental tool to study mechanisms underlying the complex processes of organogenesis, as well as the maturation of the main functions during an organism's development. Thus, this PhD utilized the benefits offered by state-of-the-art molecular tools such as the Fluidigm Biomark Dynamic Array technology, which allows expression analysis of multiple genes on multiple samples.

More comprehensive insight and understanding regarding early larval development, from a morphological and a complementary molecular point of view, is of major importance for future sustainable aquaculture of economically important fish species, where it is necessary to better define rearing and feeding conditions. As such, in order to molecularly understand phenotypic sensitivity to extrinsic environmental factors (such as temperature and salinity) as well as essential functionality of processes relating to feeding, this PhD followed expression of targeted genes controlling the underlying mechanisms during the larval yolk-sac stages and the transition from endogenous to exogenous feeding in European eel.

3. Objectives

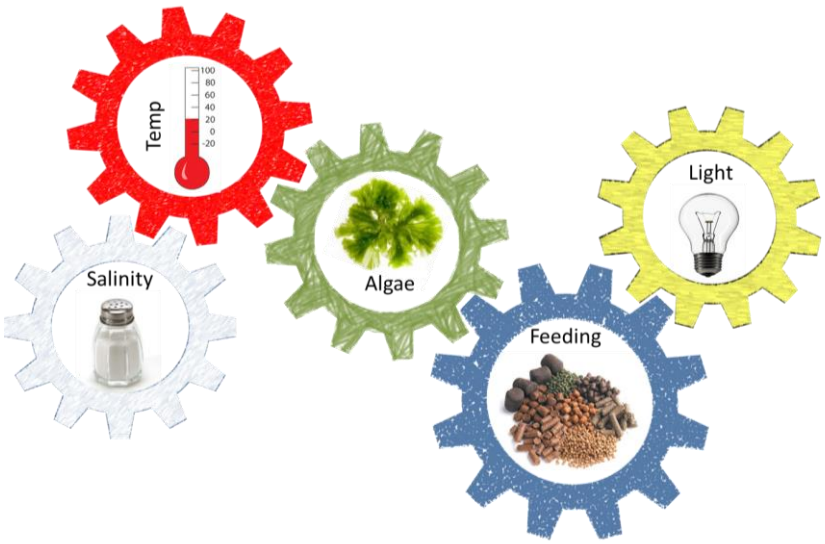

Figure 10: PhD schematic overview: biotic and abiotic factors influencing European eel early life history.

The aim of this PhD thesis is to fill the gaps in knowledge about the early ontogeny and physiology of European eel larvae with emphasis on biotic and abiotic factors influencing early life stages in order to improve rearing conditions that would lead to increasing amounts of first-feeding larvae and enable successful transition to exogenous feeding during European eel larviculture (***Fig. 10***). Hence, the main objectives of this PhD project were to identify optima and tolerance limits for temperature and salinity as well as requirements related to feeding initiation in larval culture using molecular tools to link morphological observations to the underlying mechanisms.

Temperature (Studies 1-3)

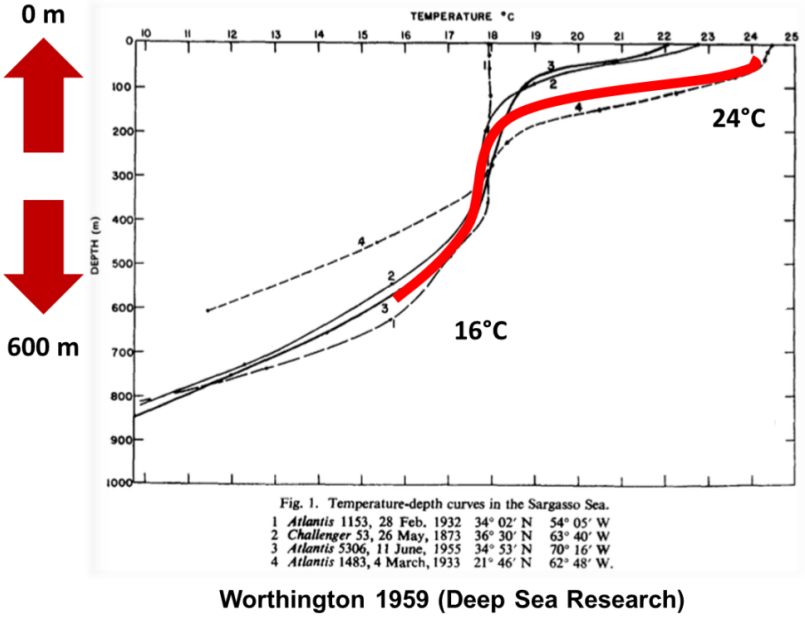

Figure 11: Schematic of the thermal range occurring between the Sargasso Sea surface and 600m depth, representing the temperature regime used in our experiment.

The first experiment addressed adequate temperature regimes for larval culture. As such, the temperatures occurring at different depths in the assumed spawning area of the Sargasso Sea were experimentally resembled in order to indicate the possible thermal tolerance range and limits for development and growth of European eel offspring (***Fig. 11***). This information was used to define five different thermal regimes within the experimental set-up, forming the basis of three different studies. Here, **Study 1** aimed at identifying the optimal thermal range for healthy development by following temperature-dependent morphology and the expression patterns of genes relating to stress response as well as growth and development throughout early larval ontogeny. In a

similar context, **Study 2** specifically followed the expression of genes relating to the thyroid hormone signaling pathway that is known to regulate growth, development, and metabolism in vertebrates and to elucidate the sensitivity of this mechanism to temperature. Furthermore, **Study 3** shed light onto the molecular ontogeny of the immune system during larval development by considering temperature as an immunomodulatory factor, in order to better understand the high mortality commonly occurring during early life history as well as the associated vulnerability to pathogens and extrinsic environmental factors (such as temperature), which is of importance both for aquaculture production and natural recruitment.

Salinity (Study 4)

Thereafter, research focused on optimizing the rearing environment during culture conditions by identifying the range of tolerated and suited salinities. Thus, it was hypothesized that decreasing salinity would enable the reduction of metabolic demands and facilitate efficient use of energy and conversion of endogenous resources into somatic development during European eel pre-leptocephalus larval culture. Inspired by the Japanese eel research, the goal was to ease osmotic "stress" by reducing salinity to iso-osmotic levels, aiding an osmotic balance between plasma osmolality and the aquatic environment in order to optimize conditions for healthy development and enhanced survival of European eel larvae. Again, the goal was to complement morphological findings by investigating the interlinked underlying molecular mechanisms. Thus, **Study 4** followed expression profiles of genes related to processes involved in or affected by osmoregulation, such as ion transport, energy metabolism, thyroid metabolism and stress response in order to complement morphological aspects such as larval biometry (morphology and growth), deformities and survival.

Nutrition (Studies 5-6)

Presently, experience in European eel larviculture is limited and natural feeding regimes of early leptocephali still remain enigmatic. Thus, initial tests of potential food items focused on exploring what the first-feeding larvae would be interested in. Here, **Study 5** was an early pilot study aiming at determining whether specific dietary regimes, chemo-attractants, and environmental light conditions impact the incidence of first-feeding, gut fullness and behavior in European eel pre-leptocephalus larvae reared in captivity. Leaps later, **Study 6** aimed at molecularly identifying the "first-feeding window" as well as exploring how nutritional supplementation (green-water) prior to first-feeding and the actual ingestion of food affect larval morphological development. This study included analyses of the hormonal and enzymatic regulation of feeding by following the expression of genes relating to appetite, food intake, digestion, energy metabolism as well as growth and thyroid metabolism. In addition, the larval nutritional status was assessed *via* individual nucleic acid analysis during the transition from endogenous towards and throughout exogenous feeding in European eel larvae.

4. Filling gaps in knowledge

Study 1

Temperature effects on gene expression and morphological development of European eel, *Anguilla anguilla* larvae

This study identified the thermal tolerance range as well as limits and preferences for optimized development and growth of artificially produced European eel offspring.

Temperature is known to be of major importance for optimization of rearing conditions in aquaculture, as it controls fundamental biochemical processes, thereby influencing developmental rates and survival of fish larvae (O'Connor et al., 2007). In the closely related Japanese eel, early ontogeny was found to be strongly influenced by temperature, with optimum thermal conditions (~25°C) similar to those found in their recently identified natural spawning area (Tsukamoto et al., 2011; Ahn et al., 2012). In this regard, European eel larvae are believed to initiate their migration journey from the Sargasso Sea, a water mass characterized by a rather constant salinity of 36.5 psu, a seasonal thermocline (~18°C) at a depth of ~300 m and a warm water (20-28°C) upper layer zone (Worthington 1959; Castonguay and McCleave 1987). As such, the experimental design of this study addressed a thermal range, approximately representing the temperatures occurring between the Sargasso Sea surface and 600m depth (***Fig. 11***). For a simplified schematic of the experimental set-up please see ***Fig. 12***.

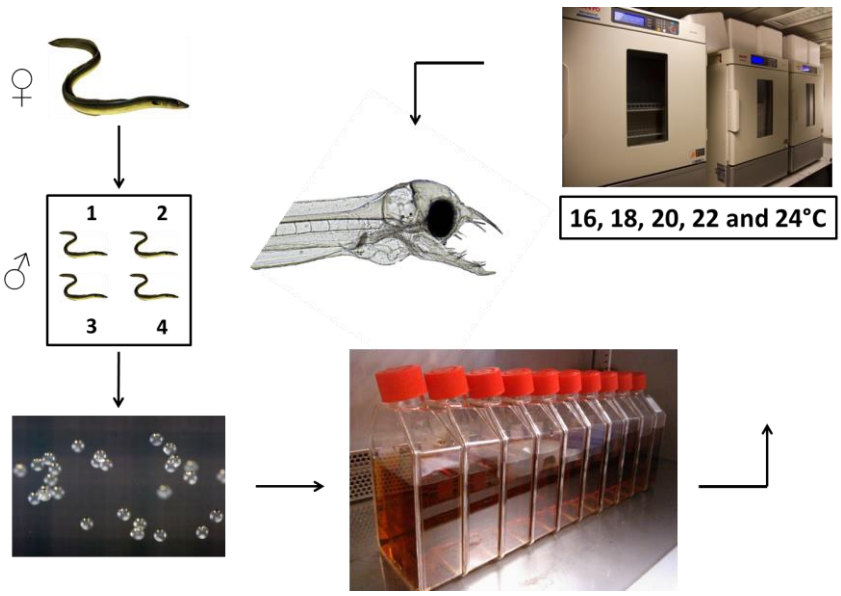

Figure 12: Simplified schematic of the experimental set-up. Embryos and larvae, from each parental cross, were reared in experimental flasks containing antibiotics (in thermal controlling incubators) at five temperatures (16, 18, 20, 22 and 24 ± 0.1°C).

Temperature was found to influence all traits investigated, providing important insights on thermal phenotypic sensitivity and the underlying gene expression of the molecular mechanisms relating to stress response [heat shock protein (*hsp*)] as well as growth and development [growth hormone (*gh*) and insulin-like growth factor (*igf*)] in European eel larvae. Moreover, we observed a reduced larval stage duration at higher temperatures due to accelerated development, where the higher the temperature, the earlier the expression response of any specific targeted gene. Larvae generally developed and grew throughout ontogeny until the first-feeding stage by utilizing their yolk reserves in all temperature treatments,

except at 24°C which was found to be the deleterious upper thermal limit. The thermal tolerance limits identified in this study might not only advance the rearing conditions for future culture of European eel larvae but possibly also contribute to the understanding for further hypotheses regarding the natural spawning conditions and location. As such, this study suggests that the habitat (or niche) of the earliest life stages of European eel in nature might be the characteristic "18°C" ocean layer of the Sargasso Sea. However, extrapolation of lab work to the field should always be approached carefully. Thus, the mystery of the exact location of European eel early life stages and their preferred conditions in nature still remain an enigma. Additionally, the results clearly showed that increasing temperature had a deleterious impact on European eel embryonic and larval survival, further supporting the recent hypotheses, that rising temperatures in the Sargasso Sea might show negative effects on eel recruitment.

To summarize, from an aquaculture perspective this study determined the thermal tolerance limits and identified a more optimal intermediate thermal environment, with efficient growth and fewer deformities as well as high growth hormone (*improved growth*) and low heat shock protein (*decreased stress*) expression, for future rearing of early life history stages of European eel. In conclusion, understanding the biological responses, limits and adaptabilities or preferences to extrinsic environmental factors, such as temperature, provides enhanced knowledge for the optimization of rearing techniques of this socially and economically important species, as well as insights into its ecology.

Study 2

Temperature induced variation in gene expression of thyroid hormone receptors and deiodinases of European eel (*Anguilla anguilla*) larvae

This study enhanced our knowledge regarding the underlying thyroid hormone (TH) signaling pathway, which is a molecular mechanism controlling European eel early life development.

Generally, THs are key regulators of growth, development, and metabolism in vertebrates (Power et al., 2001; Warner and Mittag, 2012; Tata, 2006), while increasing evidence suggests that they play important roles during early life development and metamorphosis in fish (Marchand et al., 2004; Walpita et al., 2007; Infante et al., 2008; Campinho et al., 2010). TH is produced in the thyroid gland (or thyroid follicles) mainly as T4 (thyroxine), which is then metabolized by deiodinase (DIO) enzymes in peripheral tissues, whereas their action is mostly exerted by binding to a specific nuclear thyroid hormone receptor (THR). The Japanese conger eel was previously chosen as model species to investigate the TH role on development and metamorphosis of Anguilliformes (Kawakami et al., 2003). Thereafter, an investigation of *thrs* showed differentially regulated gene expression during development and metamorphosis in Japanese eel (Kawakami et al., 2013).

Thus, it was of importance to further elucidate the thyroid hormone signaling pathway during the most sensitive period of anguillid early life history, but especially during the still mysterious early life history of European eel. In this study, we i) cloned and characterized *thr* sequences, ii) investigated the expression pattern of the different subtypes of *thrs* and *dios*, and iii) studied how temperature affects the expression of those genes in artificially produced early life history stages of European eel, reared in different thermal regimes (16, 18, 20 and 22°C) from hatch until first-feeding.

First, this study identified two isoforms of *thra* (*thraA* and *thraB*) and two isoforms of *thrb* (*thrbA* and *thrbB*) which showed high similarity to other mammalian, bird, amphibian or fish species, but with highest similarity to the closely related Japanese eel. Moreover, we generally observed that the warmer the temperature the earlier the expression response of a specific target gene. More specifically, in real time, the expression profiles appeared very similar and only shifted with temperature, while in developmental time, expression of all genes differed across selected developmental stages, such as hatching, during teeth formation or at first-feeding. Within the thermal tolerance limits, we commonly observed elevated expression of all deiodinases (*dio1*, *dio2* and *dio3*) towards and within the first-feeding window, which probably corresponds to a timing of refinement for organogenesis (e.g. brain, liver, gastro-intestinal tract, etc.) and/or specific functional tissue (e.g. teeth, eyes, etc.), in order to ensure optimal transition from endogenous to exogenous feeding.

In conclusion, all genes investigated in this study, involved in the mediation of TH action, were significantly affected (in real and/or developmental time) by larval age, temperature, and/or their interaction. Thus, we demonstrate that *thrs* and *dios* show sensitivity to temperature and are involved in and during early life development of European eel.

Study 3

Larval Fish Immunity at Variable Temperatures

This study shed light on the molecular ontogeny of the larval immune system under different thermal scenarios and linked immune gene expression to commonly observed mortality during European eel early life history.

In almost all fish species natural mortality is highest during early life history, where subtle differences in survivorship can cause large variability in offspring production (Houde, 2008). This is especially important for species that spawn (or can be artificially matured) only once in a lifetime, such as the European eel, as survival during early-life represents a substantial component of variation in lifetime fitness. An increased understanding of the physical and biological factors that influence mortality rates during these 'critical' developmental stages can enable aquaculture hatchery production, enhance recruitment predictions for fisheries and aid in the conservation of this critically endangered species (Jacoby and Gollock, 2014).

Teleost fish were the first phylogenetic group of organisms to develop an immune system that possess both the innate and adaptive arm of the immune response, characteristic to higher vertebrates (Uribe et al., 2011). However, evidence has accumulated that newly hatched fish larvae are particularly sensitive to pathogens as their immune system is not fully developed during the first weeks of life, during which they solely rely on the innate arm of the immune system, whilst exposure to pathogens intensifies due to hatching, mouth opening, and first-feeding (Magnadóttir et al., 2004; Vadstein et al., 2013; Ferraresso et al., 2016). Knowledge of the development of the immune system is hence needed to design preventative methods against pathogen associated losses in aquaculture hatcheries and to better understand immune responses in variable aquatic ecosystems.

Moreover, temperature is a fundamental modulator of the immune system of fish (Bowden, 2008) and has been shown to affect immunity during fish early life history (Dios et al., 2010). The consideration of temperature as an immunomodulatory factor is therefore not only important in the development of hatchery technology in order to optimize eel rearing protocols, but also in the light of environmental changes in the natural habitat of the early life history stages of this species, where ocean warming may influence the recruitment of the critically endangered European eel (Friedland et al., 2007; Bonhommeau et al., 2008).

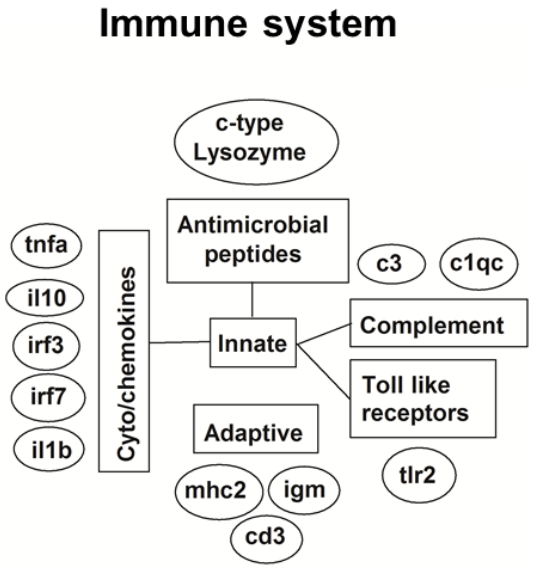

Figure 13: Schematic overview of the targeted genes associated to the innate and adaptive arm of the immune system in European eel larvae.

As such, in this study we explored the molecular ontogeny of both the innate and the adaptive immune response during early European eel larval

development (***Fig. 13***) and investigated the interaction of immune response related gene expression with temperature during early life history. Larvae were reared at four temperatures, spanning their thermal tolerance range (16, 18, 20, 22°C) and expression patterns of 11 immune genes were analyzed throughout development, from hatch until reaching the first-feeding stage. At the larvae's optimum temperature (18°C), which was revealed by **Study 1**, the pattern of immune gene expression revealed an immunocompromised phase between hatch and teeth-development (0-8 dph), caused by a lag period between initial protection and development of inherent immune competence. Additionally, at the lower end of the thermal spectrum (16°C) immune competency appeared reduced, whilst close to the upper thermal limit (22°C) larvae showed signs of thermal stress.

In conclusion, this study highlighted the influence of immune gene expression on larval survival in European eel, where we identified an immunocompromised phase during which mortality is high and larvae are more vulnerable to pathogen infection. We are confident that future research will identify this as a more general effect in marine fish larvae. In addition, we were able to demonstrate the influence of temperature on larval immune gene expression close to their upper and lower thermal limit. These findings have important implications on rearing conditions and disease prevention protocols (e.g. timing of vaccination, immunostimulation treatments) of European eel in culture and on our understanding of ocean warming impacts on fish recruitment.

Study 4

Salinity reduction benefits European eel larvae: Insights at the morphological and molecular level

This study identified optimized rearing conditions towards an iso-osmotic environment that enables the reduction of metabolic demands, while facilitating the efficient conversion of endogenous energy supplies into somatic growth during European eel pre-leptocephalus larval culture.

Most fish species are hyper-osmotic in freshwater, where plasma osmolality is higher than the environment and hypo-osmotic in seawater, where plasma osmolality is lower than the environment (Marshall and Grosell, 2005). Thus, in freshwater they need to actively take up ions to counteract the diffusive ion loss and osmotic water gain, while in seawater they need to maintain osmotic balance through a desalting process to counteract osmotic water loss (Evans, 2008). Eels are euryhaline species that have adapted to cope with both, hyper- and hypo-osmotic environments, likely due to regular salinity changes in their habitats and migrations between freshwater and marine environments at different developmental stages within their life cycle (Tesch, 2003). Eel offspring naturally occur in a hypo-osmotic environment in the ocean (Castonguay and McCleave, 1987), but interestingly, it was shown that reducing salinity during early life history rearing under culture conditions, results in better growth and survival of Japanese eel larvae (Okamura et al., 2009).

As such, this study investigated how different salinity reduction scenarios (***Fig. 14***) affect European eel larval biometry (such as morphology and growth), deformities and survival. Moreover, this study took advantage of the European eel genome that was recently sequenced and assembled, offering new perspectives for eel research regarding the molecular biology of this species. Thus, we followed expression profiles of genes related to processes involved in or affected by osmoregulation, such as ion and water transport [$Na^+K^+2Cl^-$ cotransporters (*nkcc*), aquaporins (*aqp*)], energy metabolism [mitochondrial ATP Synthase F0 subunit 6 (*atp6*), cytochrome C oxidase 1 (*cox1*)], thyroid metabolism [thyroid hormone receptors (*thr*), deiodinases (*dio*)] and stress response [heat shock proteins (*hsp*)].

		Age in days post hatch (dph)												
Reduction		0	1	2	3	4	5	6	7	8	9	10	11	12
dph	psu day^{-1}	Salinity (psu)												
Control		36												
0	1	35	34	33	32	31	30	29	28	27	26	25	24	23
	2	34	32	30	28	26	24	22	20	18	16			
	4	32	28	24	20	16								
3	1	36			35	34	33	32	31	30	29	28	27	26
	2	36			34	32	30	28	26	24	22	20	18	16
	4	36			32	28	24	20	16					

Figure 14: Overview of seven different salinity treatments. Larvae were reared at 36 psu (control) and in six further scenarios, where salinity was reduced on 0 or 3 days post hatch and at rates of 1, 2 or 4 psu/day towards iso-osmotic conditions.

When reducing salinity towards iso-osmotic conditions, results showed an improved growth and an amazing 4-fold increase in survival, while eel larvae were able to keep energy metabolism related gene expression (*atp6, cox1*) at stable levels. As such, when reducing salinity, an energy surplus associated to reduced osmoregulation demands and stress (lower *nkcc*, *aqp* and *hsp* expression), likely facilitated the observed increased survival, improved biometry and enhanced growth efficiency. Additionally, the salinity reduction decreased the amount of severe deformities such as spinal curvature and emaciation but on the other hand induced an edematous state of the larval heart, resulting in the most balanced survival/deformity ratio when salinity was decreased on 3 dph and at 2 psu/day.

In conclusion, this study clearly showed that salinity reduction regimes towards iso-osmotic conditions facilitated the European eel pre-leptocephalus development and revealed the existence of highly sensitive and regulated osmoregulation processes at such early life stage of this species. Hence, the overall knowledge gained from this study adds to our understanding of underlying biological and physiological mechanisms during early life history of European eel and provides a promising step in the strive for sustainable aquaculture of this species.

Study 5

First-feeding by European eel larvae: A step towards closing the life cycle in captivity

This study identified benchmark diets, optimized feeding conditions and described behavioral feeding patterns never before observed in first-feeding European eel.

Even though breeding European eel in captivity is a complex task, recent advances in assisted reproduction and culturing techniques have allowed mass production of high-quality yolk-sac (pre-leptocephalus) larvae (Tomkiewicz, 2012; Butts et al., 2014; Politis et al., 2014; Sørensen et al., 2016a,b), expanding the focus of experimental research to include larval performance in first-feeding trials. When initiating first-feeding, fish larvae detect prey *via* a wide range of chemical (olfaction and taste), visual and physical (mechanical) stimuli (Rønnestad et al., 2013). For instance, natural or synthetic chemo-tactic stimulants attract larvae from a distance, foster the appropriate orientation, and promote the initiation of prey capture and ingestion (Kamstra and Heinsbroek, 1991; Reig et al., 2003; Barroso et al., 2013). In the case of Japanese eel, larvae are attracted to and successfully feed on a slurry diet based on shark egg yolk (Tanaka et al., 2003), hen egg yolk or exoskeleton-free Antarctic krill (Okamura et al., 2013), or a protein hydrolysate-based diet (Masuda et al., 2016). Additionally, a minute illoricate rotifer (*Proales similis*) was suggested as an alternative diet closer to the natural larval trophic levels (Wullur et al., 2013). Unfortunately, suitable start feeds for cultured larval European eel were not previously identified, while larval-prey interactions in nature are still not completely clear (Riemann et al., 2010).

Thus, this field warranted immediate investigation, where this study tested specifically tailored diets (with and without natural chemo-attractants), under light and no-light conditions, for initiation of larval ingestion rate, gut fullness, and behavioral patterns.

Results showed up to 50% of cultured larvae ingesting a diet composed of enriched rotifers, concentrated and emulsified into a paste (***Fig. 15***), with or without natural feeding stimulants. Moreover, we show that first-feeding eel larvae are able to execute a complex goal-oriented motor response, where we observed highly distinctive modes of swimming from short-term bouts, slow steady-state cruising to quick lunges for either prey attacks or spontaneous escape behaviors. Overall, swimming activity increased over the duration of the experiment, co-varied with

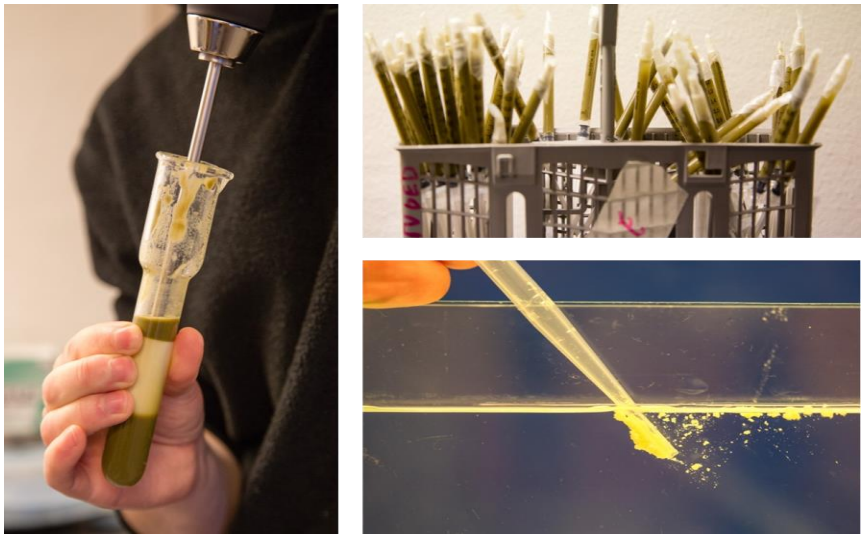

Figure 15: Manufacturing enriched rotifer paste

the frequency of attacks and increased in the presence of live rotifers (***Fig. 16***) or chemo-attractants, probably by increasing the awareness of food availability. We also detected improved ingestion at higher light intensities, suggesting that eel larvae are visual feeders and probably explaining the large eye globules. However, the observed chemo-attraction and successful food intake with and without light, indicates that this species is able besides using visual cues, to also utilize other stimuli (olfaction and taste) to detect prey.

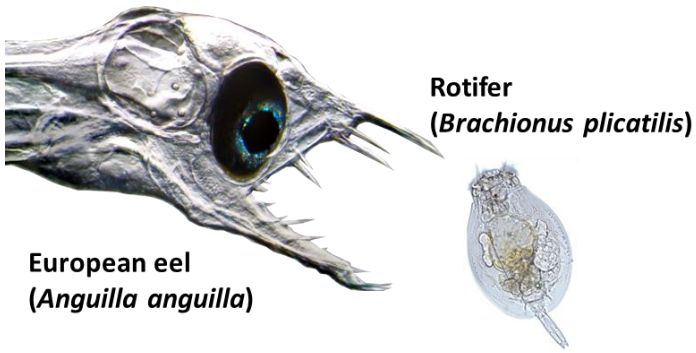

Figure 16: Schematic of European eel larvae attacking a rotifer.

In conclusion, this study documented the first evidence of first-feeding European eel larvae reared in captivity, moving this field one step closer towards understanding an undisclosed phase in the European eel life cycle, which is the transition from newly hatched endogenous feeding pre-leptocephalus larvae to the exogenous leptocephalus stage. Together, this work provided benchmark diets and conditions for future feeding and growth trials.

Study 6

Nutritional condition and molecular ontogeny of first-feeding European eel larvae

This study shed light on the species specific molecular ingestion and digestion potential and timing in order to understand the distinct nutritional predisposition and the capacity for adaptation towards utilizing dietary components.

Establishment of culture techniques throughout the larval stage until metamorphosis is still challenged by lack of insights especially regarding dietary requirements for the unique pre-leptocephalus larvae. The nutritional requirements of fish larvae are species specific and even differ across developmental stages within a species, mainly due to the major morphological and physiological changes during ontogeny (Zambonino-Infante and Cahu, 2001). Several scientists have focused on identifying natural larval eel feeding resources, but despite the increasing insight the natural first-feeding regimes of pre-leptocephali still remain an enigma. Increased scientific inquiry has also been subjected towards identifying potential first-feeding diets for laboratory reared eel larvae in aquaculture where the first exogenously feeding (Japanese) eel larvae was reported two decades ago (Tanaka et al., 1995). Unfortunately, identifying suitable feeds for larval European eel has been rather stagnant for several decades and only recently (in **Study 5**) it was documented that artificially produced *A. anguilla* pre-leptocephali successfully ingested a diet based on rotifers (*Brachionus plicatilis*) with or without natural chemo-attractants.

Since European eel research succeeded in producing larvae which are able to exogenously feed, the opportunity has emerged to elaborate our knowledge on the nutritional condition of individual larvae *via* nucleic acid (RNA/DNA) content analysis (Clemmesen, 1993) and to examine key physiological mechanisms regulating feeding, digestion and growth. As such, we here reared European eel larvae with or without the presence of algae (*Nannochloropsis*, *Pavlova* and *Tetraselmis*) from 0 to 14 dph and with or without the presence of food (rotifer paste) from 15 dph and onwards. For a simplified schematic of the experimental set-up please see ***Fig. 17***.

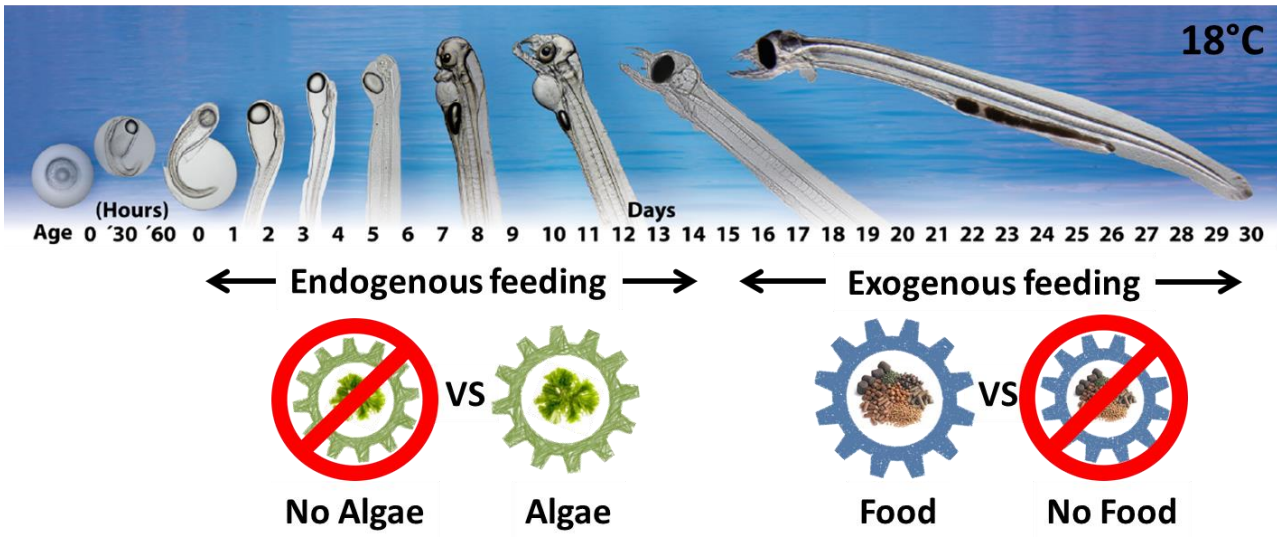

Figure 17: Schematic of experimental set-up. Artificially produced European eel larval morphological and molecular performance was compared under clear or green-water conditions during endogenous feeding (0-14 days post hatch) and under feeding or non-feeding conditions during exogenous feeding (15 days post hatch and onwards).

We then measured larval biometrics, quantified individual larval nucleic acid contents and followed mRNA expression patterns of selected genes relating to some of the most important mechanisms during early life history and transition from endogenous to exogenous feeding in European eel larvae. The up-regulated expression of genes encoding appetite stimulators (*ghrelin*) and inhibitors (*cholecystokinin*) on 12 dph, indicated the beginning of the first-feeding window, but no significant benefit of algal presence (green-water) was observed. Moreover, mRNA expression patterns of selected genes encoding some of the most important digestive enzymes relating to protein (*trypsin*), lipid (*triclyceride lipase*) and carbohydrate (*amylase*) hydrolysis revealed the essential digestive ontogenetical processes occurring from 14 to 20 dph. On 15 dph, eel larvae were first fed a paste consisting of enriched rotifers (as described in **Study 5**) and on 16 dph, a molecular response to initiation of exogenous feeding was observed in the expression pattern of genes relating to energy metabolism, food intake, growth and thyroid metabolism. Moreover, a significantly higher RNA content in feeding compared to non-feeding larvae was observed, which clearly indicates increased metabolic activity associated to protein synthesis. Additionally, an increased DNA content in feeding compared to non-feeding larvae was observed, which in combination with the increased amount of RNA and greater body area observed, reflected a higher growth pattern in feeding larvae. However, RNA:DNA ratios still decreased from 12 dph onwards (irrespective of initiation of feeding), indicating a generally low larval nutritional condition, probably leading to the unavoidable "point-of-no-return" and subsequent irreversible mortality due to unsuccessful utilization of exogenous feeding.

In conclusion, the here applied nutritional regime facilitated a short-term benefit, where feeding European eel larvae were able to sustain growth and better condition than their non-feeding conspecifics. Moreover, our study revealed that the success of exogenous feeding in European eel larvae, occurs concurrently with the onset of a broad array of genetically pre-programmed underlying molecular factors, which are known to regulate physiological functions of feeding. Thus, the knowledge gained constitutes essential information in order to develop efficient feeding strategies for European eel larvae and will hopefully provide a promising step towards sustainable aquaculture of this species.

5. Conclusion and future perspectives

Not too long ago larviculture of European eel was considered almost impossible. However, the recent advances in assisted reproduction have enabled European eel captive breeding, with consecutive promising steps towards increased viable offspring production and first attempts to culture larvae. Taking larval culture one step further, this PhD within the EEL-HATCH project has explored unknown territories within European eel research by morphologically and molecularly defining physiological thermal and osmoregulatory tolerance limits and preferences, leading to the identification of improved conditions for early life rearing and resulting in increased amounts of stronger larvae reaching the first-feeding stages. This enabled emerging feeding trials, which resulted in documented first evidence of first-feeding European eel larvae reared in captivity and thus moved eel research one step closer towards understanding the transition from newly hatched endogenous feeding pre-leptocephalus larvae to the exogenous feeding leptocephalus stage, which is an undisclosed phase of the European eel life cycle in nature.

Temperature

The research conducted within this PhD project, outlined the thermal tolerance limits for European eel early life history, providing new knowledge for optimized rearing conditions in eel hatcheries but may also contribute to further hypotheses regarding the natural spawning conditions and location. As such, the new insight provided may inspire the planning of new sampling locations during future Sargasso Sea eel cruises. Here, a temperature of 24°C was found to be the deleterious upper thermal limit, indicating that the upper surface layer of the Sargasso Sea would be too warm for development of the earliest eel stages, while the optimum temperature of 18°C suggests a deeper environmental niche as probably previously anticipated. However, extrapolation of lab work to nature should always be approached carefully, especially considering the fact that eel eggs and embryos are positively buoyant and that eel larvae may vertically migrate already at an early stage in a dynamic oceanic environment, meaning that they may encounter different thermal regimes at daily or even hourly intervals. Moreover, the suggested optimum thermal range most probably represents the favorable conditions for the stenothermal development of the embryonic stages and thus the thermal preferences for later European eel larval stages should be addressed in order to precisely define optimized future larviculture procedures. Furthermore, future investigations should address temperature as a modulatory factor during induction of gametogenesis but also document the transgenerational thermal plasticity of offspring which will be influenced by the environmental experience of the parental generation.

Salinity

Thereafter, this PhD morphologically and molecularly elucidated osmoregulation related processes and identified an optimized rearing environment for European eel pre-leptocephalus larvae by decreasing salinity towards iso-osmotic conditions. However, we also demonstrated that reducing salinity was a tradeoff process, which majorly improved survival and growth but also induced an edematous state of the larval heart. Hence, future research needs to address the persistency of the

edematous state of the larval heart and if or how it represents an obstacle in further larval development. Moreover, future rearing units and protocols need to be modified in order to facilitate the gradual salinity change, but it would also be beneficial to identify methodological alternatives that facilitate the decreased physiological demands and thus the increased survival and growth potential, but at the same time eliminate morphological deformities that hinder early life development.

Nutrition

Furthermore, within the studies of this PhD we did not observe any significant benefit of "green-water" during European eel pre-leptocephalus rearing, neither morphologically nor molecularly, despite that it was proven to be beneficial during comparable developmental stages in other fish species (Reitan et al., 1997; Cahu et al., 1998; Lazo et al., 2000). This could potentially be due to the fact that the frozen algae species used in this study do not represent the appropriate choice for triggering the desired effect of earlier maturation in digestive functionality. However, besides providing a direct nutritional supply and an indirect stimulation of appetite or digestive function, the presence of live algae can have a probiotic effect, influencing the bacterial community of the rearing water and thus, the establishment of an early gut microbial flora in fish larvae (Vadstein, 1993; Skjermo and Vadstein, 1993). A similar effect has been observed in the case of dietary addition of lactic acid bacteria, which benefitted fish larvae by facilitating increased larval growth and decreased developmental deformities during early ontogeny of sea bass, *Dicentrarchus labrax* (Lamari et al., 2013). Moreover, application of nutritional supplements such as probiotics, have received increasing attention in aquaculture, as it has been suggested that they show a protective action on the intestinal mucosal cells, stimulating the innate immune response and thus causing an elevated state of immuno-readiness in fish such as tilapia, *Oreochromis niloticus* (Standen et al., 2013). As such, we strongly encourage future investigations to address the potential of nutritional supplementation on influencing the bacterial flora of the water and the microbial gut colonization, in order to facilitate an earlier and improved larval digestion potential as well as increased development and growth also in European eel.

Now that European eel larvae are ingesting feed in captivity, the next step will be to establish a dietary composition and regime that ensures high growth rates and survival, which will resolve one of the major 'bottlenecks' in the process of establishing on-growing larval culture throughout the leptocephalus stage and thus reaching the phase of transformation to glass eels. To achieve this, a series of studies should be conducted to enhance knowledge on dietary requirements of European eel larvae. This includes a thorough examination of the ontogenetic development of the digestive tract morphology and a comprehensive analysis of pancreatic and intestinal enzymatic activity, as well as their corresponding gene expression. The capacity of larvae to digest and absorb various nutrients should also be investigated *via* a technique called "force-feeding", whereby *in vivo* studies target digestibility and assimilation of key nutrients using radiolabeled dietary nutrients. Based on attained insights, an assortment of feeds (formulated or live) could be manufactured to ensure adequate nutrition throughout the larval stage.

Molecular tools

Last but not least, this PhD took advantage of the available European eel genome which was recently sequenced and assembled. However, the fragmented nature of the current eel genome assembly does not presently allow taking full advantage of the genomic and transcriptomic possibilities. Thus, it will be of great advantage if future research would focus on providing a complete overview of the European eel genome, enabling more possibilities for genomic and transcriptomic studies, which will provide a more detailed understanding of molecular processes underlying biological functions in this species. Considering the already available tools and the amazingly fast pace of progression within this sector, this should not be a real hurdle. Moreover, this PhD provided important insights into molecular processes during early life history of European eel by using molecular tools, but it can often be difficult to disentangle complex interactions. Despite the increased understanding gained by the findings of this PhD, there are still plenty more exciting possibilities for future research based on application of available tools but also rapidly improving new methods. The continuous technological advancements especially regarding development of bio-informatics and molecular tools are increasingly enabling more detailed insight into underlying molecular mechanisms of biological processes and expand the scientific questions that research is and will be able to address, especially when genetics are coupled to traditional disciplines. We are confident that even though the world of genetics has already transformed as fast as no research sector before, the opportunities have only started to unfold.

In conclusion, all the above mentioned exciting future research should be within our grasp and together will provide invaluable new information regarding the species biology and ecology, that can probably contribute to solving the mystery of the European eel spawning location but most importantly will move the entire eel aquaculture research field closer towards completing the life cycle in captivity of this socially and economically important as well as critically endangered fish species.

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Study 1:

Temperature effects on gene expression and morphological development of European eel, *Anguilla anguilla* larvae

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RESEARCH ARTICLE

Temperature effects on gene expression and morphological development of European eel, *Anguilla anguilla* larvae

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Abstract

Temperature is important for optimization of rearing conditions in aquaculture, especially during the critical early life history stages of fish. Here, we experimentally investigated the impact of temperature (16, 18, 20, 22 and 24˚C) on thermally induced phenotypic variability, from larval hatch to first-feeding, and the linked expression of targeted genes [heat shock proteins (*hsp*), growth hormone (*gh*) and insulin-like growth factors (*igf*)] associated to larval performance of European eel, *Anguilla anguilla*. Temperature effects on larval morphology and gene expression were investigated throughout early larval development (in real time from 0 to 18 days post hatch) and at specific developmental stages (hatch, jaw/teeth formation, and first-feeding). Results showed that hatch success, yolk utilization efficiency, survival, deformities, yolk utilization, and growth rates were all significantly affected by temperature. In real time, increasing temperature from 16 to 22˚C accelerated larval development, while larval gene expression patterns (*hsp70*, *hsp90*, *gh* and *igf-1*) were delayed at cold temperatures (16˚C) or accelerated at warm temperatures (20–22˚C). All targeted genes (*hsp70*, *hsp90*, *gh*, *igf-1*, *igf-2a*, *igf-2b*) were differentially expressed during larval development. Moreover, expression of *gh* was highest at 16˚C during the jaw/teeth formation, and the first-feeding developmental stages, while expression of *hsp90* was highest at 22˚C, suggesting thermal stress. Furthermore, 24˚C was shown to be deleterious (resulting in 100% mortality), while 16˚C and 22˚C (~50 and 90% deformities respectively) represent the lower and upper thermal tolerance limits. In conclusion, the high survival, lowest incidence of deformities at hatch, high yolk utilization efficiency, high *gh* and low *hsp* expression, suggest 18˚C as the optimal temperature for offspring of European eel. Furthermore, our results suggest that the still enigmatic early life history stages of European eel may inhabit the deeper layer of the Sargasso Sea and indicate vulnerability of this critically endangered species to increasing ocean temperature.

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Introduction

European eel (*Anguilla anguilla*) aquaculture is capture-based, relying on wild-caught juvenile glass eels entering coastal waters, which are farmed until marketable sizes. However, historically low stock levels and failing recruitment [1] render this practice unsustainable and therefore establishment of breeding technology and larvi-culture is required for future aquaculture of this critically endangered species [2]. By, modifying the hormonal treatments from Japanese eel (*Anguilla japonica*) protocols, recent advances in assisted reproduction of European eels have led to a stable production of eggs and larvae, forming the basis of development of larval culture technology and first-feeding protocols [3]. In order to establish another promising step towards sustainable aquaculture of this species, it is necessary to identify optimal rearing conditions for early life history (ELH) stages.

The choice of rearing conditions used in aquaculture for a particular species is commonly based on either ambient conditions in their natural environment or experimental findings. In the case of European eel, the natural environmental regimes of embryos and the earliest larval stages (pre-leptocephalus) remain unclear. The spawning area of this species has been delimited to the western Atlantic Ocean (Sargasso Sea) and validated by the occurrence of the so far earliest larval stages found in nature [4–6]. Thus, eel larvae are believed to initiate their migration journey from the Sargasso Sea, a water mass characterized by a rather constant salinity of 36.5 ppt, a seasonal thermocline (~18˚C) at a depth of 200–300 m and a warm water (20–28˚C) upper layer zone [6, 7]. Thereafter, towards the European continent, glass eels inhabit temperate regions of a wide range of latitudes and longitudes with temperatures spanning between 2 and 28˚C [8, 9].

In the past decades, there has been an increasing interest in eel research, especially concerning assisted reproduction and subsequent ELH rearing conditions in aquaculture. Thus, breeding protocols using assisted reproduction were developed for the Japanese eel in the 1970s [10], leading to the first glass eel production in recent years [11]. Since then, several studies have focused on optimizing rearing conditions of Japanese eel larvae, including the identification of salinity regimes or thermal tolerance ranges and limits during ELH stages [12–15]. In more detail, it was shown that a 50% reduction of the original seawater salinity resulted in increased offspring growth and survival performance [12]. Additionally, early ontogeny was found to be also influenced by temperature, showing suboptimal performance towards colder and warmer temperature limits (16–31˚C), with optimum thermal conditions (~25˚C) similar to those found in the recently identified natural spawning area of the Japanese eel [15, 16]. Moreover, induced maturation, *in vitro* fertilization, and early development of the American eel (*Anguilla rostrata*) have been reported, with larvae surviving up to 6 days post-hatch (dph) when reared at 20˚C [17]. Furthermore, eel hybrids have gained attention as an alternative for the difficult assisted reproduction practices in aquaculture. Hybrids between *A*. *anguilla* and *A*. *rostrata* occur naturally since they share spawning grounds [18], though hybrids between male *A*. *anguilla* and female *A*. *japonica* have also been experimentally produced and their endogenously feeding larvae developed for 9 dph at 21–22˚C [19]. Captive breeding has also been attempted with the long-finned (*A*. *dieffenbachii*) and short-finned (*A*. *australis*) eels. Though, successful hatching has only been reported for *A*. *australis* or the *A*. *australis* × *A*. *dieffenbachii* hybrid, under a thermal regime of 18.2–22.7˚C [20]. Moreover, production of hybrid larvae from male *A*. *anguilla* and female *A*. *australis* and their survival for up to 5 dph was reported when fertilized and reared at 20–21˚C [21]. Nevertheless, the thermal tolerance ranges of all the above species remain to be further investigated.

Identifying the optimal thermal conditions for rearing of eel ELH stages and establishing hatchery practice will benefit the future aquaculture industry, commonly targeting high

production efficiency where high survival and growth potential are fundamental to a cost-effective production. Relatively small changes in rates of growth and mortality during embryogenesis and/or larval ontogeny can significantly influence reproductive success [22] and thus production efficiency in aquaculture. Temperature controls fundamental biochemical processes, thereby influencing developmental rates and survival of marine fish larvae [23]. Especially during early development, teleost offspring can be stenothermal and be profoundly affected by even minor temperature changes [24, 25]. Moreover, their ELH stages are influenced physiologically by extrinsic factors such as temperature and their interaction with intrinsic properties endowed to them by their parents [24, 26].

Today molecular methods and tools are increasingly accessible and economically affordable. Whole species genomes are publically available, including the European eel genome, which was recently sequenced and assembled [27–28]. This offers new perspectives for eel research, such as using RNA sequencing to identify actors involved in different biological processes, cellular components and molecular functions that are of importance in this species [29]. As such, in order to molecularly understand phenotypic sensitivity of ELH stages to extrinsic environmental factors (such as temperature), it is now possible to follow expression of targeted genes controlling the development of this fish species [30, 31]. For instance, heat shock proteins (HSP) play a fundamental role in the regulation of normal protein synthesis within the cell and are critical to the folding and assembly of other cellular proteins [30]. As such, it is hypothesized that an up-regulated expression of fish larval *hsp*'s, would be associated with vulnerability to thermal injury, as the *hsp* response is a cellular mechanism activated to prevent cell damage caused by thermal stress [32]. Moreover, fish communicate with their physiologic environment, by using the somatotropic axis, a mechanism combining growth hormones (GH) and the closely connected and regulated by GH, insulin-like growth factors (IGF), that are involved in most physiological processes including metabolism and growth [31]. Here, it is hypothesized that the activation of the GH/IGF system triggering cell proliferation and DNA synthesis, could be stimulated by temperature and the up-regulated expression response would be associated among others with improved growth, metabolism and development [31, 33].

In this context, we experimentally investigated the impact of temperature on European eel larvae from hatching to first-feeding, through an integrative morphological and molecular approach. Thus, the objectives of this study were i) to identify the thermal tolerance range and limits for development and growth of European eel larvae, and ii) to elucidate thermally induced phenotypical changes and the interlinked gene expression of genes (*hsp's*, *gh* and *igf's*) involved in molecular mechanisms commonly associated to fish early life development.

Materials and methods

Ethics statement

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2012-15-2934-00458). Briefly, adult eels were anesthetized using ethyl p-aminobenzoate (benzocaine) before tagging and handling. Endogenously feeding larvae of European eel were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222). All efforts were made to minimize animal handling and stress.

Broodstock management and gamete production

Female silver eels were obtained from a freshwater lake, Vandet, Jutland, Denmark. Male eels were obtained from a Danish commercial eel farm (Stensgård Eel Farm A/S). Experimental

maturations were conducted at a DTU Aqua research facility at Lyksvad Fishfarm, Vamdrup, Denmark, where eels were housed in 300 L tanks equipped with a recirculation system [34]. Eels were maintained under low intensity light (~20 lux), 12 h day/12 h night photoperiod, salinity of ~36 ppt, and temperature of 20˚C. Acclimatization took place over 10 days. As eels naturally undergo a fasting period from the onset of the pre-pubertal silvering stage, they were not fed during treatment. Prior to experimentation, eels were anaesthetized (ethyl p-aminobenzoate, 20 mg L^{-1}; Sigma-Aldrich, Missouri, USA) and tagged with a passive integrated transponder. Females used for experiments (n = 4) had a mean (± SEM) standard length and body weight of 65 ± 4 cm and 486 ± 90 g, respectively. To induce vitellogenesis females received weekly injections of salmon pituitary extract (Argent Chemical Laboratories, Washington, USA) at 18.75 mg kg^{-1} body weight [11, 34]. To stimulate follicular maturation and induce ovulation, females received 17α,20ß-dihydroxy-4-pregnen-3-one (Sigma-Aldrich, Missouri, USA) at 2 mg kg^{-1} body weight [35] and were strip-spawned within the subsequent 12–14 h. Male eels (n = 11) had a mean (± SEM) standard length and body weight of 40 ± 3 cm and 135 ± 25 g, respectively. Males received weekly injections of human chorionic gonadotropin (Sigma-Aldrich, Missouri, USA) at 150 IU per fish [34]. Prior to fertilization, an additional injection was given and milt was collected by strip-spawning ~12 h after administration of hormone. Milt samples were pipetted into a P1 immobilizing medium [36] and only males with sperm motility of category IV (75–90%) were used for fertilization within 4 h of collection [37]. Only floating viable eggs/embryos were further used for experimentation.

Experimental conditions

The experiment was repeated 4 times, within the same spawning season (2015), each time using a different parental cross. Eggs from each female were "crossed" with a sperm pool of several males to experimentally create 4 parental crosses. Eggs from each female were stripped into dry 36 × 30 × 7 cm plastic containers and gametes were swirled together while 0.2 μm filtered UV sterilized seawater was added for a gamete contact time of 5 min [37]. Seawater was obtained from the North Sea (~32.5 ppt) and temperature was adjusted to 20˚C (± 0.1˚C) while salinity was adjusted to 36 (± 0.1) ppt using Red Sea Salt (Red Sea Europe, Verneuil-sur-Avre, France) as previously defined [37, 38]. Egg density was determined by counting 3 × 0.1 mL subsamples of the floating layer. Within 30 min post fertilization, ~500 floating viable eggs/embryos per 100 mL, with a mean size (± SD) of 1.5 ± 0.1 mm (measured from images taken at 2 hpf), were distributed in replicated 600 mL flasks [182.5 cm^2 sterile tissue culture flasks with plug seal caps (VWR®)] dedicated to larval sampling for morphology (3 replicates) and molecular analysis (2 replicates). Additionally, ~500 floating viable eggs/embryos per 100 mL were distributed in replicated (3×) 200 mL flasks (75 cm^2 non-pyrogenic and non-cytotoxic flasks with plug seal caps, Sarstedt, Inc.) dedicated to sampling for hatch success and deformities at hatch. Seawater from the North Sea (0.2 μm filtered and UV sterilized), was adjusted to 36 (± 0.1) ppt (as above) and supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) to increase survival as previously defined [39]. Embryos and larvae, from each parental cross, were reared in thermal controlling incubators (MIR-154 Incubator, Panasonic Europe B.V.) at five temperatures (16, 18, 20, 22 and 24˚C ± 0.1˚C). All experimental units were acclimatized to the treatment temperature within 1 h and salinity was kept at 36 (± 1) ppt. Temperature and salinity conditions of treatments were chosen to closely resemble the environmental conditions encountered at different depths of the assumed spawning areas in the Sargasso Sea [6]. Rearing of embryos and larvae took place in darkness while handling and sampling under low intensity (< 2.2 μmol m^{-2} s^{-1}) light conditions as previously defined [40].

Data collection

Larval development and gene expression were followed from hatch until the corresponding first-feeding stage in each temperature treatment. The first-feeding stage, as previously defined [3], was set as the time point when eye pigmentation, mouth and jaw formation was completed. Endogenously feeding larvae of European eel were anesthetized using tricaine methanesulfonate (MS-222) 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-Fi1, Nikon Corporation, Japan) attached to an objective microscope (Eclipse 55i, Nikon Corporation, Japan). NIS-Elements D analysis software (Version 3.2) was used to analyze the images of eggs, embryos, and larvae (Nikon Corporation, Japan).

Hatch success and deformities. Once hatching was completed, embryos and larvae within each experimental unit, dedicated to analysis of hatch success and deformities (× 3 replicates × 4 parental crosses) from each temperature treatment were digitally imaged for later classification. Embryos that were oversized, dark, discolored or exhibited abnormities in the cytoplasm were considered dead. Hatching success was then expressed as the total number of larvae divided by the total number of eggs. Larvae with abnormal and/or malformed head, body, yolk-sac or tail regions were classified as deformed.

Biometry. For analysis of larval morphology, ~15 larvae (× 3 replicates × 4 parental crosses) from each temperature were randomly sampled at hatch and every second day post-hatch. Larvae were digitally imaged for later analyses, where total length (from the tip of the snout to the posterior end of the caudal fin) and total yolk-sac area were measured from each larva. Larval growth and yolk utilization (YU) were measured from the change in length and yolk area from hatching until first-feeding. Yolk utilization efficiency (YUE) was measured by dividing the increase in length from hatching until first-feeding by the corresponding decrease in yolk area.

Molecular analyses. For molecular analysis, ~30 larvae (× 2 replicates × 4 parental crosses) from each temperature (16, 18, 20 and 22˚C) were randomly sampled at hatch and every second day post-hatch until the first-feeding stage. Those larvae were not observed under the microscope (to avoid any influence on gene expression) but were immediately euthanized using MS-222, rinsed with deionized water, preserved in a RNA Stabilization Reagent and kept at -20˚C following the procedure suggested by the supplier (Qiagen, Hilden, Germany).

For total RNA extraction, the larval pool (~30 larvae) of each replicate was homogenized in 800 μl Tri-Reagent (Sigma-Aldrich, Missouri, USA). After obtaining the aqueous phase by incubation in 160 μl chloroform, RNA was extracted using the InviTrap® Spin tissue RNA MiniKit (STRATEC Biomedical AG, Berlin-Buch, Germany) following the manufacturer's instructions. RNA concentration (764 ± 60 ng μl^{-1}) and purity (260/280 = 2.12 ± 0.16, 230/260 = 2.16 ± 0.16) were determined by spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany). From the resulting total RNA, 680 ng were transcribed using the Quanta qScript cDNA Synthesis Kit (Promega, Germany) according to the manufacturer's instructions, including an additional gDNA wipe out step prior to transcription [Quanta PerfeCta DNase I Kit (Promega, Germany)].

The *ef1a*, *18s*, *40s* genes were chosen as housekeeping genes since 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 [41]. The expression levels of target (*gh*, *igf-1*, *igf-2a*, *igf-2b*, *hsp70*, *hsp90*) and reference (*ef1a*, *18s*, *40s*) genes were determined by quantitative real-time PCR (qRT-PCR), using specific primers. Primers were designed using primer 3 software v 0.4.0 (http://frodo.wi.mit.edu/primer3/) based on

predicted or cloned cDNA sequences available on Genbank databases (Table 1). Predicted cDNA sequences for *gh*, *igf-2a*, *igf-2b* and *18s* genes were deduced from genomic DNA sequences originated from the European eel genome [27]. All primers were designed for an amplification size ranging from 75 to 200 nucleotides.

Expression of genes in each larval sample (2 biological replicates) were analysed in technical triplicates of each primer using the qPCR Biomark^{TM} HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips) as previously described [42]. 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 μl cDNA per sample at 10 min at 95˚C; 14 cycles: 15 s 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 and measured using the ΔΔ Ct method [43]. Coefficient of variation (CV) of triplicates was calculated and checked to be < 0.04 [41]. If CV was found to be > 0.04, triplicates were checked for outliers and if possible duplicate measurements were used. If the use of duplicates was not possible ($CV > 0.04$) the whole data point was omitted from the analysis. In this automated analysis, malfunctions on the chip and/or unsuccessful RNA extractions can result in expression failures, as was the case in the Day 14 sample of 18˚C in this study. Since qBase+ software revealed that *ef1a*, *18s* and *40s* mRNA levels were stable throughout the analyzed samples ($M < 0.4$), those genes were chosen as housekeeping genes.

Statistical analyses

All data were analyzed using SAS statistical software (version 9.1; SAS Institute Inc., Cary, North Carolina). Residuals were tested for normality using the Shapiro–Wilk test and

Table 1. Sequences of European eel (*Anguilla anguilla*) primers used for amplification of genes by qRT-PCR. Primers were designed from predicted or cloned cDNA sequences available in Genbank nucleotide and WGS databases. The table lists accession number and corresponding database of target gene sequences.

Full name	Abbreviation	Databases	Accession Numbers	Primer sequence (5'_3') (F: Forward; R: Reverse)
Heat Shock Protein 70	*hsp70*	GenBank	AZBK01685255	F: TCAACCCAGATGAAGCAGTG
				R: GCAGCAGATCCTGAACATTG
Heat Shock Protein 90	*hsp90*	GenBank	AZBK01838994	F: ACCATTGCCAAGTCAGGAAC
				R: ACTGCTCATCGTCATTGTGC
Growth Hormone	*gh*	GenBank WGS	AZBK01601863	F: TGAACAAGGGCATCAATGAA
				R: CGGAGCTTTCTCACATCCTC
Insulin-like Growth Factor-1	*igf-1*	GenBank Nucleotide	EU018410.1	F: TTCCTCTTAGCTGGGCTTTG
				R: AGCACCAGAGAGAGGGTGTG
Insulin-like Growth Factor-2-1	*igf-2a*	GenBank WGS	AZBK01717674	F: ACAACGGATATGGAGGACCA
				R: GGAAGTGGGCATCTTTCTGA
Insulin-like Growth Factor-2-2	*igf-2b*	GenBank WGS	AZBK01622663	F: AAAGCTTTGGGACAGCTTCA
				R: CGCAGCTGTGTACGTGAAAT
Elongation Factor 1-alpha	*ef1a*	GenBank Nucleotide	EU407824.1	F: CTGAAGCCTGGTATGGTGGT
				R: CATGGTGCATTTCCACAGAC
Ribosomal 18S RNA	*18s*	GenBank WGS	AZBK01681648	F: AGAGCAGGGGAACTGACTGA
				R: ACCTGGCTGTATTTGCCATC
Ribosomal 40S RNA	*40s*	GenBank TSA	GBXM01005349.1	F: TGACCGATGATGAGGTTGAG
				R: GTTTGTTGTCCAGACCGTTG

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homogeneity of variances was tested using a plot of residuals versus fit values (PROC GLOT, SAS Institute 2003). Data were $\log_{10}$ or arcsine square-root-transformed when data deviated from normality and/or homoscedasticity [44].

Larval hatch success, survival, deformities, growth rate, yolk utilization, and yolk utilization efficiency. The effect of temperature on larval hatch success, survival, deformities, growth rate, yolk utilization, and yolk utilization efficiency was determined using a series of one-way ANOVA models, where parental cross was considered a random factor (SAS PROC MIXED; SAS Institute 2003). Tukey's post-hoc analyses were used to compare least-squares means between treatments.

Larval morphology and molecular analyses. Statistical models were used to investigate temperature effects on larval morphology and gene expression throughout early larval development (Ages 0 to 18 dph) and at specific developmental stages (Stages 1–3). Across the different temperature treatments, Stage 1 represents the day of hatch, Stage 2 represents the timing of jaw/teeth formation and Stage 3 represents the first-feeding stages. Together this allowed us to decipher changes in temperature at standardized and real-time developmental intervals.

To examine the effect of temperature on larval morphology and gene expression throughout early development, we used two statistical approaches. In the first approach, we analyzed the data using a series of repeated measures mixed-model ANOVAs (PROC MIXED; SAS Institute 2003). Models contained temperature (16, 18, 20, 22 and 24˚C) and age (0 to 18 dph) or stage (1, 2 and 3) main effects as well as the temperature × age (or stage) interaction. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate [45]. Temperature and age (or stage) were considered fixed, whereas parental cross was considered random. Tukey's post-hoc analyses were used to compare least-squares means between treatments. If a significant temperature × age (or stage) interaction was detected, the model was decomposed into a series of reduced ANOVA models to determine the effect of temperature for each age (or stage) and of age (or stage) for each temperature. This was the case for length, yolk area, *gh*, *igf-1*, *hsp70* and *hsp90*. The reduced models involved only preplanned comparisons and did not include repeated use of the same data, so a-level corrections for a posteriori comparisons were not necessary.

In the second approach, we examined variation in larval morphology and gene expression, throughout development at each temperature, by fitting either linear, quadratic, cubic or exponential equations to the data (PROC REG; SAS Institute 2003). This allowed us to create predictive models to explore the shape of developmental variation for each temperature. Linear, quadratic, cubic and exponential equations were chosen a-priori to fit the data based on the available literature [46, 47]. Final equation selection (linear, quadratic, cubic or exponential) was based on an F-statistic: $d.f._j \times (R^2_j - R^2_i) / (1 - R^2_j)$, where: R^2_i = the R^2 for the i-th order, R^2_j = the R^2 for the next higher order, $d.f._j$ = the degrees of freedom for the higher-order equation with j degrees of freedom in the numerator and $d.f._j = n - j - 1$ degrees of freedom in the denominator [47]. Graphs and regressions were prepared in SigmaPlot (Version 13.0).

Results

Larval development, hatch success, survival and deformities

Generally, development was delayed when embryos and larvae were reared in cold temperatures while accelerated in warm temperatures (Fig 1). Developmental rates were similar across all parental crosses investigated and larvae reached the first-feeding stages within 8 days or 232 hours post-fertilization (hpf) at 22˚C compared to 10 days (288 hpf) at 20˚C, 12 days (344 hpf) at 18˚C or 16 days (456 hpf) at 16˚C. Larval hatch success (Fig 2A) did not differ between 16,

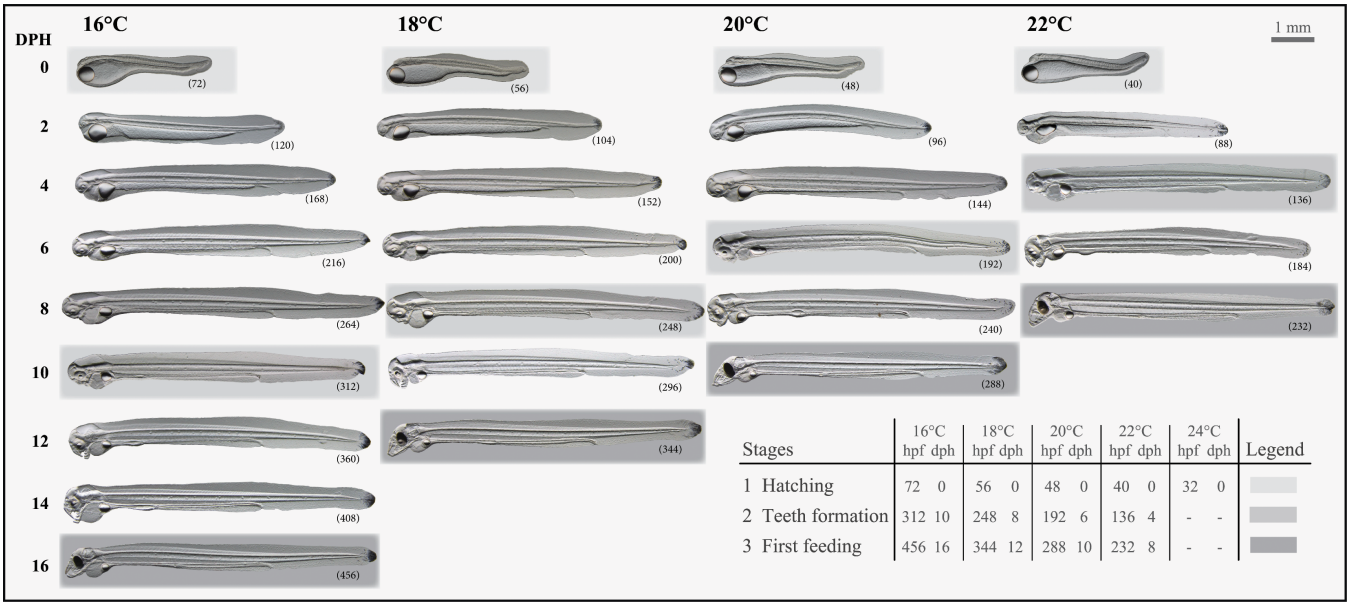

Fig 1. Timing of morphological features during development of larval European eel (*Anguilla anguilla*). Larval age presented in hours post-fertilization (hpf) and days post-hatch (dph) for five rearing temperature regimes. Across the different temperature treatments, Stage 1 represents the day of hatch, Stage 2 represents the timing of jaw/teeth formation, while Stage 3 represents the first-feeding stages.

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18 and 20˚C but significantly decreased at 22˚C ($P < 0.0001$), while only two (in total) larvae hatched at 24˚C, but they died shortly after hatch (100% mortality). Larval survival, in terms of longevity (Fig 2B), was lowest at 22˚C (4 ± 2 dph) and highest at 18˚C (14 ± 2 dph). Temperature had a significant influence on the incidence of larval deformities at hatch ($P < 0.0001$), where larvae reared at 18˚C showed significantly less deformities (24 ± 6%), compared to 16˚C (48 ± 6%), 20˚C (44 ± 6%) or 22˚C (90 ± 6%) (Fig 2C).

Larval growth, yolk utilization and yolk utilization efficiency

Temperature had a significant influence ($P < 0.0001$) on larval growth rate. Here, larval growth increased with increasing temperature from 0.27 ± 0.02 at 16˚C to 0.41 ± 0.02 mm d^{-1} at 22˚C (Fig 2D). Similarly, temperature had a significant impact ($P < 0.0001$) on YU, which increased with increasing temperature, from 0.04 ± 0.003 mm^2 d^{-1} at 16˚C to 0.08 ± 0.003 mm^2 d^{-1} at 22˚C (Fig 2E). Temperature did not significantly influence YUE which decreased with increasing temperature, from 0.66 ± 0.02 mm^2 d^{-1} at 16˚C and 18˚C to 0.62 ± 0.02 mm^2 d^{-1} at 22˚C (Fig 2F).

Larval length

Significant differences in length between temperatures occurred at all developmental stages investigated ($P < 0.0001$), where larvae reared at 22˚C were smaller compared to all the other temperature treatments (Fig 3A). Larvae reared at 16, 18 and 20˚C did not differ in length in Stage 3 (first-feeding), though significant differences occurred at the earlier developmental Stages (1 and 2). In real time, we observed a temperature × age interaction ($P < 0.0001$), thus the model was decomposed into a series of reduced ANOVA models to determine the effect of temperature for each age (Fig 3B) and of age for each temperature (Fig 3C–3F). Significant differences in length among temperatures occurred throughout development on 0, 2, 4, 6, 8, 10, 12 and 14 dph ($P < 0.003$). Typically, larvae reared in the colder (16˚C) and warmer (22˚C)

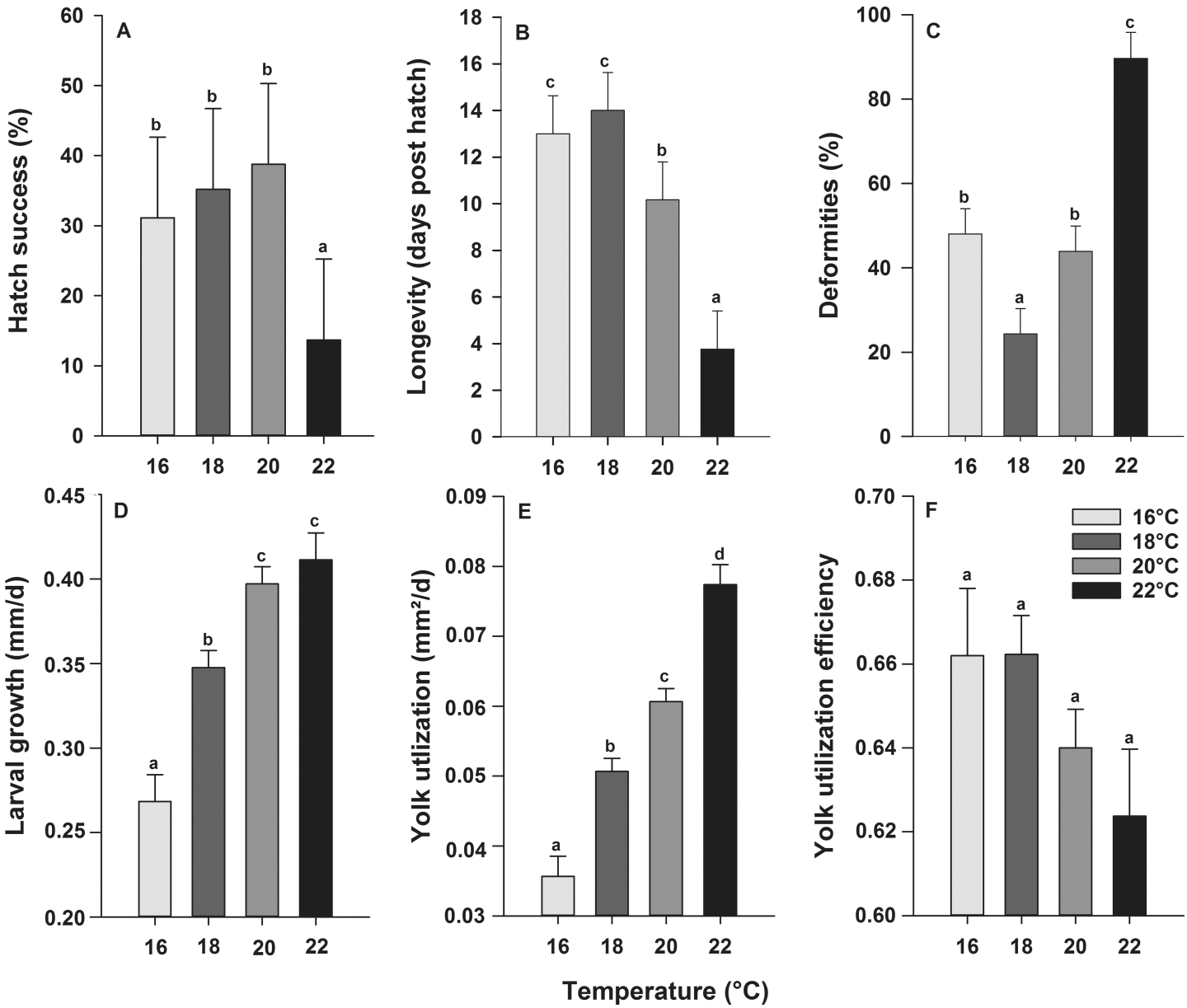

Fig 2. Effect of rearing temperature on larval European eel (*Anguilla anguilla*) (A) hatch success, (B) survival, (C) deformities at hatch, (D) growth rate, (E) yolk utilization and (F) yolk utilization efficiency. Values represent means (± SEM) among four crosses at each temperature. Means were contrasted using the Tukey-Kramer method and treatments with the same letters are not significantly different (P > 0.05).

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thermal treatments were significantly smaller than larvae reared in the intermediate temperatures of 18 and 20˚C. Larvae reared at 16˚C grew to sizes similar to larvae reared at 18˚C or 20˚C (14–18 dph). Within each temperature, larval age significantly influenced length when larvae were reared at 16, 18, 20 and 22˚C ($P < 0.0001$). Relationships between age and larval length can be explained by exponential regressions (B-E) at 16˚C [$y = 6.85 * \exp(-\exp(-(x + 0.63) / 3.54))$, $R^2 = 0.99$], 18˚C [$y = 7.24 * \exp(-\exp(-(x + 0.45) / 2.94))$, $R^2 = 0.99$], 20˚C [$y = 7.23 * \exp(-\exp(-(x + 0.53) / 2.79))$, $R^2 = 0.99$] and 22˚C [$y = 6.19 * \exp(-\exp(-(x + 0.22) / 1.74))$, $R^2 = 0.98$].

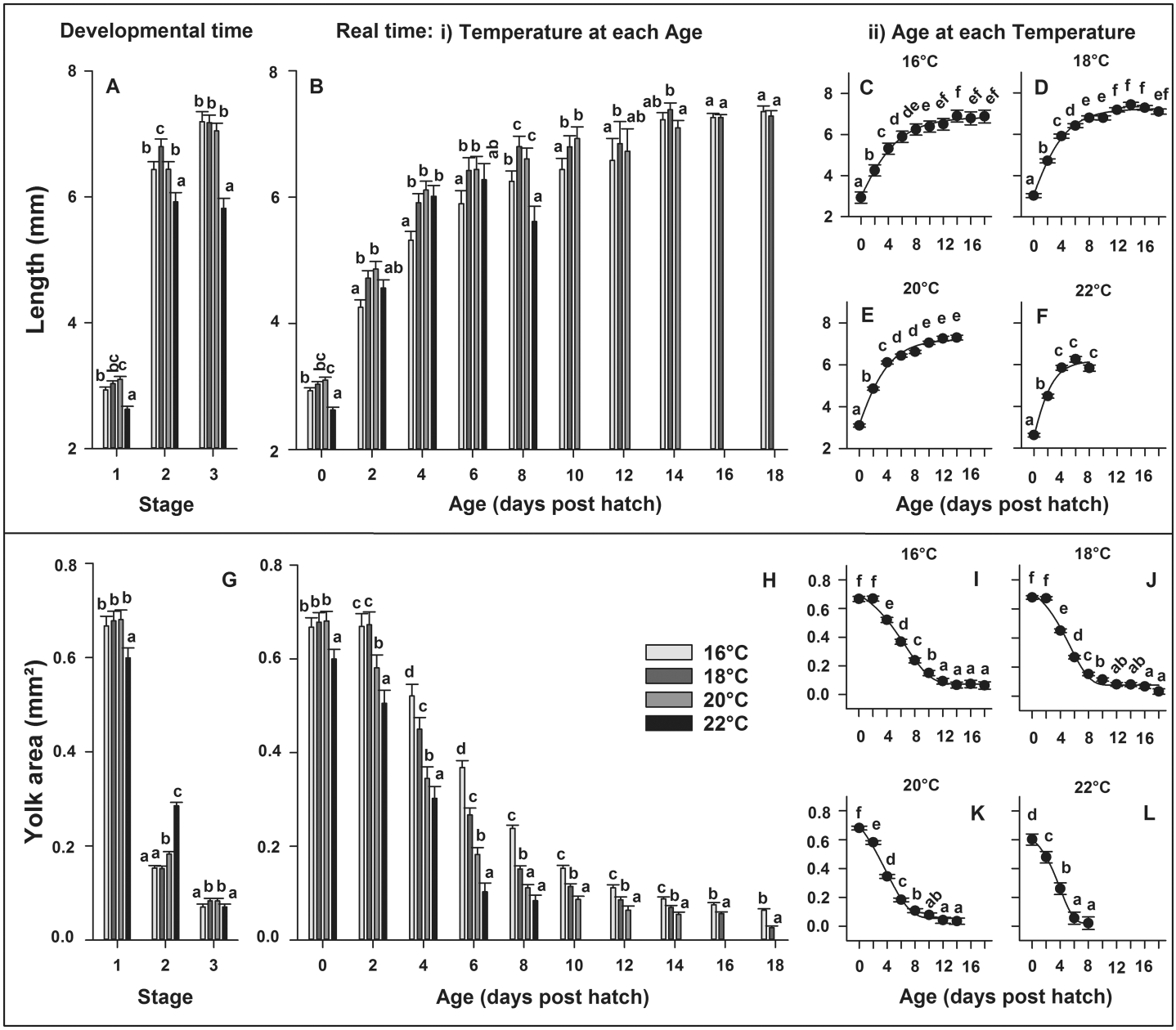

Fig 3. Effect of rearing temperature on larval European eel (*Anguilla anguilla*) length (A) or yolk area (G) at specific developmental Stages (1, 2 and 3) and length (B) or yolk area (H) in real time, as well as effect of age on length (C-F) or yolk area (I-L) at each temperature. Exponential regressions explain the relationship between age and length (C-F; $P < 0.025$, $R^2 > 0.98$) as well as between age and yolk area (I-L; $P < 0.006$, $R^2 = 0.99$) at all temperatures. Values represent means (± SEM) among four crosses at each temperature. Treatments with the same letters are not significantly different ($P > 0.05$).

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Larval yolk area

In developmental time (Fig 3G), significant differences in yolk area among temperatures occurred at all developmental stages investigated ($P < 0.008$). Larvae reared at 22˚C had most yolk reserves on Stage 2 but least yolk reserves on Stage 1 (hatch) or Stage 3 (first-feeding) compared to the other temperature treatments. Larvae reared at 16, 18 and 20˚C did not differ in yolk area on Stage 1, though significant differences occurred at the later developmental

Stages (2 and 3) investigated. In real time, yolk area was significantly affected by the temperature × age interaction ($P < 0.0001$) so the model was again decomposed to determine the effect of temperature for each age (Fig 3H) and of age for each temperature (Fig 3I–3L). Significant differences in yolk area among temperatures occurred throughout development on 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 dph ($P < 0.002$). The warmer the rearing temperature, the faster the YU and less yolk reserves were left for larvae to utilize. Within each temperature, larval age significantly influenced yolk area when larvae were reared in 16, 18, 20 and 22˚C ($P < 0.0001$). Relationships between age and yolk-sac area can be explained by exponential regressions (G-J) at 16˚C [$y = 0.07 + 0.74 * \exp(-\exp(-(x—6.60) / - 3.71))$, $R^2 = 0.99$], 18˚C [$y = 0.08 + 0.74 * \exp(-\exp(-(x– 5.36) / - 2.82))$, $R^2 = 0.99$], 20˚C [$y = 0.05 + 0.89 * \exp(-\exp(-(x—3.85) / - 3.43))$, $R^2 = 0.99$] and 22˚C [$y = 0.02 + 0.66 * \exp(-\exp(-(x—3.97) / - 1.94))$, $R^2 = 0.99$].

Molecular analyses

Gene expression of selected genes was compared across temperature treatments in real time and at specific developmental stages (developmental time). In real time, the expression of target genes was affected by both larval age and temperature (see specific genes below). Even though the expression profiles appeared very similar in real time and only shifted with temperature, in developmental time, differences occurred across developmental stages and among temperatures (see specific genes below).

Heat shock proteins. In developmental time, expression of *hsp70* significantly ($P = 0.015$) increased on Stage 3 (first-feeding) but did not significantly differ across temperatures ($P = 0.066$; Fig 4A and 4B). In real time, gene expression of *hsp70* was significantly affected by the temperature × age interaction ($P = 0.0001$; Fig 4C). Therefore, the model was, as above, decomposed. Significant differences in gene expression of *hsp70* between temperatures occurred on 6 and 8 dph ($P < 0.010$). Expression levels of *hsp70* increased up to 5-fold at the colder (16˚C) and 4-fold at the warmer (22˚C) suboptimal thermal limit, while expression levels at 18 and 20˚C remained lower than in the other temperatures throughout ontogenetic development. Larval age significantly influenced gene expression of *hsp70* when larvae were reared at 16, 18 and 20˚C ($P < 0.003$; Fig 4D–4G) and the relationships between age and *hsp70* expression were best explained by quadratic parabola regressions (D, G) at 16˚C ($y = 1.81–0.33\ x + 0.03\ x^2$, $R^2 = 0.88$) and 22˚C ($y = 2.06–0.56\ x + 0.09\ x^2$, $R^2 = 0.99$) and by cubic sigmoidal regressions (E-F) at 18˚C ($y = 0.93–0.22\ x + 0.05\ x^2 - 0.002\ x^3$, $R^2 = 0.89$) and 20˚C ($y = 1.21–0.30\ x + 0.06\ x^2 - 0.002\ x^3$, $R^2 = 0.78$).

In developmental time, expression of *hsp90* significantly increased on Stage 1 (hatch), decreased on Stage 2 and increased again on Stage 3 (first-feeding) ($P < 0.0001$; Fig 4H). Moreover, *hsp90* expression was significantly lower at 18 and 20˚C, elevated at colder and warmer temperatures and peaked at 22˚C ($P < 0.03$; Fig 4I). In real time, gene expression of *hsp90* was significantly affected by the temperature × age interaction ($P < 0.0001$) and again the model was as previously decomposed. Significant differences in gene expression of *hsp90* among temperatures occurred throughout development on 4, 6, 8, 10 and 12 dph ($P < 0.02$; Fig 4J). Expression levels of *hsp90* increased up to 3-fold at 16, 20 and 22˚C, while expression levels at 18˚C remained (similarly to *hsp70*) lower than for the other temperatures throughout development. Larval age influenced gene expression of *hsp90* when larvae were reared in 16, 18, 20 and 22˚C ($P < 0.010$; Fig 4K–4N) and relationships between developmental age and *hsp90* expression were best explained by quadratic parabola regressions at 16˚C ($y = 1.35–0.32\ x + 0.02\ x^2$, $R^2 = 0.91$) and 22˚C ($y = 1.93–0.69\ x + 0.10\ x^2$, $R^2 = 0.93$) and by cubic sigmoidal regressions at 18˚C ($y = 1.18–0.49\ x + 0.08\ x^2 - 0.003\ x^3$, $R^2 = 0.85$) and 20˚C ($y = 1.57–0.86\ x + 0.16\ x^2 - 0.01\ x^3$, $R^2 = 0.85$).

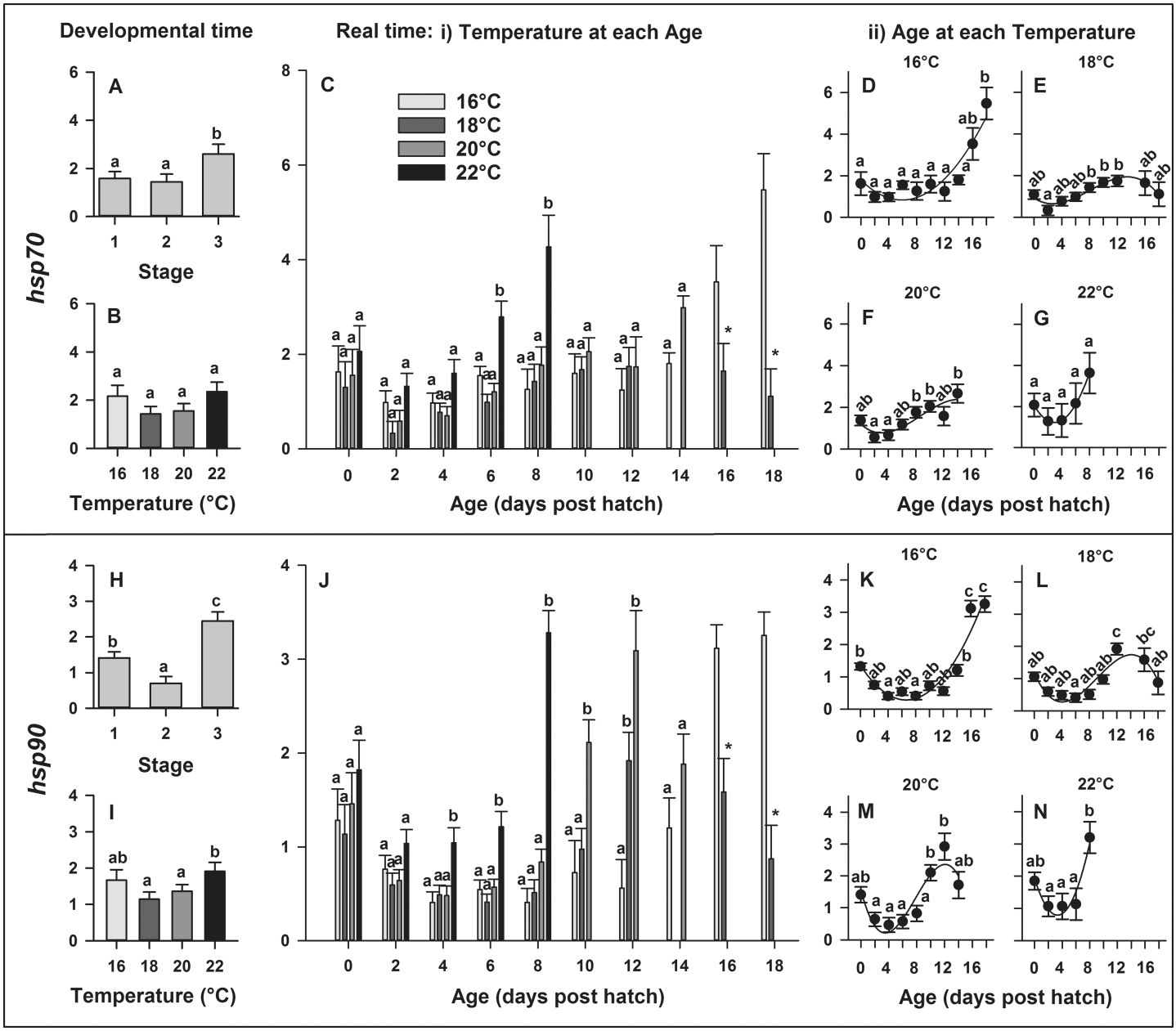

Fig 4. Effect of rearing temperature on larval European eel (*Anguilla anguilla*) gene expression of *hsp70* (A-B) or *hsp90* (H-I) at specific developmental Stages (1, 2 and 3) and *hsp70* (C) or *hsp90* (J) in real time, as well as effect of age on *hsp70* (D-G) or *hsp90* expression (K-N) at each temperature. Relationships between age and *hsp70* expression can be explained by quadratic regressions at 16 or 22˚C and by cubic regressions at 18 or 20˚C ($P < 0.03$, $R^2 > 0.78$). Relationships between age and *hsp90* expression can be explained by quadratic regressions at 16 or 22˚C and by cubic regressions at 18 or 20˚C ($P < 0.001$, $R^2 > 0.85$). Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (±SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

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Growth hormone and insulin-like growth factors. In developmental time, significant differences among temperatures occurred at Stage 2 and 3, where larvae reared at the colder thermal limit (16˚C), expressed higher levels of *gh* compared to the warmer temperatures ($P < 0.006$; Fig 5A). In real time, gene expression of *gh* was significantly affected by the temperature × age interaction ($P < 0.0001$). Thus, the model was decomposed. Significant differences in gene expression of *gh* among temperatures occurred throughout development on 2, 6, 8, 10,

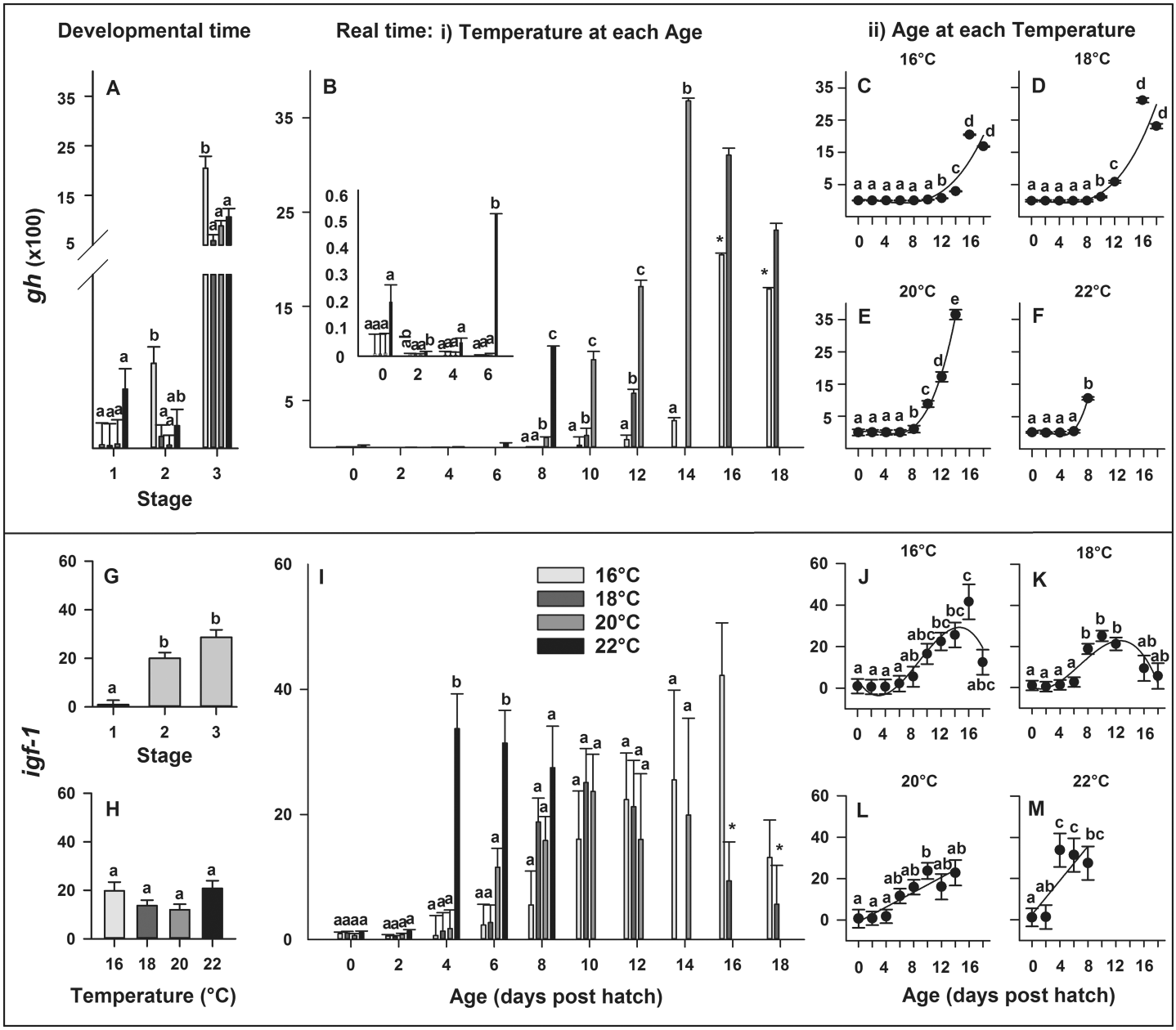

Fig 5. Effect of rearing temperature on larval European eel (*Anguilla anguilla*) gene expression of *gh* (A) or *igf-1* (G-H) at specific developmental Stages (1, 2 and 3) and *gh* (B) or *igf-1* (I) in real time, as well as effect of age on *gh* (C-F) or *igf-1* expression (J-M) at each temperature. Relationships between age and *gh* expression can be explained by cubic sigmoidal regressions at all temperatures ($P < 0.0001$, $R^2 > 0.82$). The relationship between age and *igf-1* expression can be explained by cubic sigmoidal regressions at 16 or 18˚C and by linear regressions at 20 or 22˚C ($P < 0.002$, $R^2 > 0.64$). Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (± SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

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12 and 14 dph ($P < 0.03$; Fig 5B). Expression levels of *gh* increased up to 1000-fold at 22˚C, 2000-fold at 16˚C and more than 3000-fold at 18 and 20˚C. Larval age significantly influenced gene expression of *gh* at all temperatures ($P < 0.0001$; Fig 5C–5F). Here, relationships between developmental age and *gh* expression were best explained by cubic sigmoidal regressions at 16˚C ($y = 36.21–5.24\ x—6.49\ x^2 + 0.72\ x^3$, $R^2 = 0.82$), 18˚C ($y = 40.99–23.52\ x—3.18\ x^2 + 0.75$

x^3, $R^2 = 0.86$), 20˚C (y = - 14.29 + 79.62 x—33.28 x^2 + 3.29 x^3, $R^2 = 0.99$) and 22˚C (y = 4.02 + 147.59 x—81.17 x^2 + 9.89 x^3, $R^2 = 0.99$).

In developmental time, expression of *igf-1* significantly increased with developmental stage ($P < 0.0001$; Fig 5G) but did not significantly differ across temperatures ($P = 0.081$; Fig 5H). In real time, gene expression of *igf-1* was affected by the temperature × age interaction ($P = 0.001$), thus the model was again decomposed. Differences in gene expression of *igf-1* among temperatures occurred during early development on 4 and 6 dph ($P < 0.001$; Fig 5I). Expression levels of *igf-1* increased up to 40-fold at 16˚C and 30-fold at 22˚C, indicating the colder and warmer suboptimal thermal limits respectively. Larval age significantly influenced gene expression of *igf-1* when larvae were reared in 16, 18, 20 and 22˚C ($P < 0.002$; Fig 5J–5M). Here, the relationships between developmental age and *igf-1* expression were best explained by cubic regressions at 16˚C (y = 4.18–5.53 x + 1.11 x^2 - 0.04 x^3, $R^2 = 0.82$) or 18˚C (y = 0.19–1.39 x + 0.65 x^2 - 0.03 x^3, $R^2 = 0.82$) and by linear regressions at 20˚C (y = - 0.92 + 1.79 x, $R^2 = 0.84$) or 22˚C (y = 2.45 + 4.14 x, $R^2 = 0.64$).

In developmental time, expression of *igf-2a* significantly decreased with developmental stage ($P = 0.020$; Fig 6A) but did not significantly differ across temperatures ($P = 0.061$; Fig 6B). In real time, gene expression of *igf-2a* was significantly affected by temperature ($P = 0.007$) and larval age ($P < 0.0001$), though no significant temperature × age interaction ($P = 0.1288$) was detected. Gene expression of *igf-2a* significantly increased at 22˚C (Fig 6C) and significantly decreased throughout ontogeny with increasing larval age (Fig 6D). The relationships between developmental age and *igf-2*a expression were best explained by linear regressions (Fig 6F–6H) at 18˚C (y = 0.73–0.02 x, $R^2 = 0.74$), 20˚C (y = 0.66–0.02 x, $R^2 = 0.65$) and 22˚C (y = 1.28–0.11 x, $R^2 = 0.61$), while no significant relationship between developmental age and *igf-2a* expression was detected at 16˚C (Fig 6E).

In developmental time, expression of *igf-2b* significantly increased with developmental stage ($P < 0.0001$; Fig 6A) but did not significantly differ across temperatures ($P = 0.658$; Fig 6I–6J). In real time, gene expression of *igf-2*b was significantly affected by temperature ($P < 0.001$) and larval age ($P < 0.0001$), while no interaction was detected between temperature and age ($P = 0.112$). Gene expression of *igf-2b* significantly increased at 22˚C (Fig 6K) and significantly increased throughout ontogeny with increasing larval age (Fig 6L). The relationships between developmental age and *igf-2b* expression were best explained by a quadratic regression at 16˚C (y = 0.76–0.02 x + 0.01 x^2, $R^2 = 0.95$) and by cubic regressions at 18˚C (y = 0.73–0.17 x + 0.07 x^2 - 0.003 x^3, $R^2 = 0.84$) or 20˚C (y = 1.51–0.63 x + 0.14 x^2 - 0.01 x^3, $R^2 = 0.91$), while no significant relationship between developmental age and *igf-22* expression was detected at 22˚C (Fig 6M–6P).

Discussion

This study identified the thermal tolerance range and limits of European eel early life history and elucidated thermally induced phenotypical changes as well as changes in the interlinked expression of genes associated to early development in fish. Temperature influenced all traits investigated. Larvae generally developed and grew throughout ontogeny until the first-feeding stages by utilizing their yolk reserves in all temperature treatments, except at 24˚C which was found to be the deleterious upper thermal limit. Generally, increasing temperature caused acceleration in development and the higher the temperature, the earlier the expression response of any specific targeted gene. In more detail, larval yolk utilization and growth rates increased, while yolk utilization efficiency decreased with increasing temperature. Furthermore, temperature influenced hatch success, time to hatch, deformities at hatch, larval survival and expression of targeted genes relating to larval development (*gh* and *igf*) and stress (*hsp*).

Here, the expression of targeted genes was affected by larval age/stage and temperature, as well as their interaction when compared in both, real or relative time.

Larval morphology

We observed a reduced larval stage duration with increasing temperature, where larvae reached the feeding stages within 8 days at 22˚C compared to 16 days at 16˚C; resulting in 50%

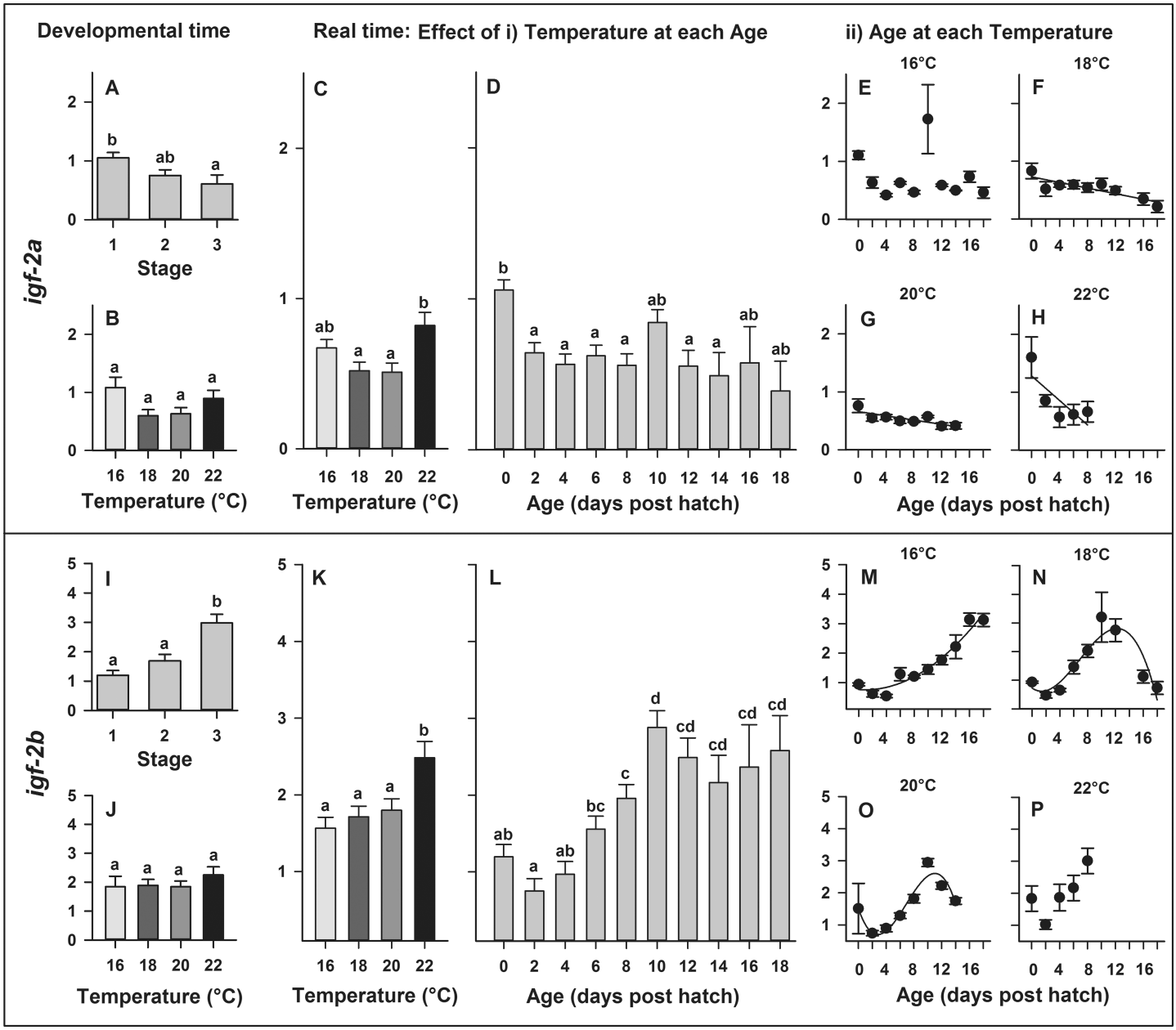

Fig 6. Effect of rearing temperature on larval European eel (*Anguilla anguilla*) expression of *igf-2a* (A-B) or *igf-2b* (I-J) at specific developmental Stages (1, 2 and 3) and i*gf-2a* (C-D) or *igf-2b* (K-L) in real time, as well as effect of developmental age on *igf-2a* (C-F) or on *igf-2b* (I-L) expression at each temperature. Relationships between developmental age and *igf-2a* expression can be explained by linear regressions at 18, 20 and 22˚C ($P < 0.025$, $R^2 > 0.61$; Fig F-H). Relationships between developmental age and *igf-2b* expression can be explained by a quadratic parabola regression (M) at 16˚C and by cubic sigmoidal regressions (N-O) at 18˚C and 20˚C ($P < 0.0001$, $R^2 > 0.84$; Fig 6I–6K). Values represent means (± SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

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faster development. A similar reduced stage duration with increasing temperature during ELH, has been previously shown in other important fish species such as haddock (*Melanogrammus aeglefinus*) [48], brown trout (*Salmo trutta*) [49], Atlantic and Baltic cod, *Gadus morhua* [50–52], Northern rock sole, *Lepidopsetta polyxystra* [53] and Atlantic herring, *Clupea harengus* [54]. Generally and within the thermal tolerance window, larval stage duration is reduced at higher temperatures due to faster growth [53, 54]. However, unfavorable thermal conditions close to the thermal tolerance limits are known to cause less efficient yolk utilization and reduced growth [25]. This phenomenon was also observed in this study, further validating the fact that increased temperature, especially towards unfavorable limits, results in less efficient conversion of yolk into somatic tissue. Moreover, we observed the lowest yolk utilization efficiency, combined with the most deformities at hatch when reared at 22˚C, pointing out an upper thermal plasticity limit. Limited thermal plasticity has been correlated with the inability to effectively make physiological adjustments to achieve homeostasis under elevated temperatures, resulting in chronic thermal stress of delta smelt, *Hypomesus transpacificus* [55]. Moreover, no larvae survived at 24˚C, representing a deleterious upper thermal limit, while larval deformities at hatch increased at 16˚C, representing a colder thermal tolerance limit during early development of European eel.

The rearing temperature (20–21˚C) used in most current protocols for hatchery production of *A*. *anguilla* larvae [3, 40], is similar to what is being used to produce hybrid larvae from male *A*. *anguilla* and female *A*. *australis* [21]. Hybrid larvae between male *A*. *anguilla* and female *A*. *japonica* have also been experimentally produced and reared at 21–22˚C [19], while the closely related *A*. *rostrata* larvae have been reared at 20˚C after assisted reproduction [17]. Moreover, an attempt to rear *A*. *australis* or *A*. *australis* × *A*. *dieffenbachii* hybrid larvae was undertaken at a thermal regime of 18.2–22.7˚C [20]. The thermal regimes for all the above mentioned eel species and their hybrid combinations seem to be rather concurrent with the thermal tolerance range identified in this study (16–22˚C). Though, we here revealed a thermal optimum of 18˚C, corresponding to the lowest incidence of larval deformities at hatch, highest larval survival and highest yolk utilization efficiency. This finding provides important information towards improving the conditions for larval rearing and production success of this species in aquaculture. Furthermore, the only eel species with a successfully closed life cycle in captivity is *A*. *japonica*, where hatchery produced larvae are reared at 25˚C [15]. This thermal optimum is far above the temperatures used for all the other eel species mentioned above, including this study, though it seems to be close to the thermal regime of the recently identified natural spawning area of this species [16].

Gene expression

In the present study, we observed an increased expression of *hsp70* and *hsp90* with increasing age and stage, while expression levels increased towards both, colder (16˚C) and warmer (22˚C) thermal limits. The expression of *hsp* has previously been linked to phenotypic variation (deformities) of green sturgeon (*Acipenser medirostris*) larvae in response to thermal stress, pointing out the importance of this cellular mechanism associating HSPs with the organism's thermal vulnerability [32]. Similarly, individual gene expression of specific molecular chaperones such as *hsp70* and *hsp47* was up-regulated in response to thermal stress, while others such as *hsp90* remained at constitutive levels across all treatments in larval delta smelt [55]. Therefore, our findings support the fact that HSP's are linked to phenotypic variation in the response and vulnerability of larvae to thermal stress. Furthermore, HSP function and response to stress is now recognized to be universal to all cells and not restricted to heat stress [30]. Nutritional status, for instance, can further affect HSP responses to thermal stress and it

has previously been shown that *hsp* expression can be related to feed deprivation in larval fish [56]. Generally, expression levels of both *hsp's* investigated in this study peaked around the first-feeding stages, potentially representing a combined effect of thermal stress and developmental preparation towards exogenous feeding. Thus, it would be interesting to further investigate this phenomenon in the future, by comparing phenotypic and molecular differences between exogenously first-feeding and starving larvae. Moreover, it would be of interest to investigate expression levels of *hsp's* when larvae are reared under different conditions and not in experimental flasks or chambers. Nevertheless though, expression levels of both genes at 18–20˚C remained lower compared to expression at the other temperatures during larval development, which in combination with occurrence of the lowest number of deformities most probably represents a more optimal thermal environment for rearing European eel offspring.

The expression of *gh*, one of the somatotropic axis actor genes, was in this study shown to be influenced by temperature during larval (pre-leptocephalus) development of European eel. It has previously been observed in fish, that GHs are involved in most physiological processes such as metabolism, and growth [31] and that they can be temperature sensitive [57]. In our study, *gh* was up-regulated and expression peaked at 18–20˚C, probably representing the most optimal environment for larval growth. Moreover, we observed an increase in *gh* expression with larval developmental age, though we did not determine the location of *gh* regulation. Expression of *gh* has been shown to be regulated in the liver of juveniles and adults, or in the brain of larval stages of gilthead sea bream, *Sparus aurata* [58]. Similarly, expression of *gh* was relatively weak during milkfish (*Chanos chanos*) embryogenesis and hatching but increased during larval development starting on day 2 post hatch, implying an endogenous production by the larval pituitary gland and coinciding with the period of accelerated larval growth [59]. The timing of ontogeny and functionality of the European eel pituitary gland has not been completely documented yet, but an immunohistochemical study in this species suggests that the majority of pituitary cells differentiate before metamorphosis to the glass eel stage, presumably during the leptocephalus stages [60]. However, the first immunohistochemical GH signal in the ricefield eel, *Monopterus albus*, has been recently detected as early as 3 dph [61]. Most likely, the sharp increase of *gh* expression observed in our study corresponds to the timing of pituitary GH secreting cells functionality. Although it needs to be further documented by immunohistological data, our results would indicate the presence of GH secreting cells during European eel early life ontogeny, much earlier than previously anticipated; an increased expression was already observed at 4–6 dph. That would also be in accordance with the results of a study of the closely related Japanese eel, where the *gh* transcripts and the production of GH protein were detected at 6 dph (when reared at 23˚C), suggesting an important role of GH in larval growth and survival before the leptocephalus stage [62].

IGF*s*, the other group of acting genes in the somatotropic axis and thus closely connected and regulated by GH, are known to be stimulated by temperature and associated among others with growth, metabolism and development [31]. In our study, we observed an increased expression of *igf-1* and *igf-2b* with increasing larval age/stage at all temperatures investigated, signifying the involvement of the somatotropic axis genes during larval development of European eel. Furthermore, transcripts for *igf's* have been detected throughout development in unfertilized eggs, embryos, and larvae of several other fish species such as Senegalese sole (*Solea senegalensis*), zebrafish (*Danio rerio*), sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and rabbitfish (*Siganus guttatus*), suggesting that they can be products of maternal as well as embryonic genomes and that *igf-1* and *igf-2* may thus regulate early development of teleosts [63–66]. Similarly, in a recent study correlating gene expression to healthy embryonic development and hatch success in European eel, it was shown that expression levels

of *igf-2* increased during embryogenesis until larval hatching [67]. Interestingly, in our study, we observed an increased expression of *igf-2a* and *igf-2b* at hatch, strengthening the theory of the involvement of *igf's* during the hatching process of European eel.

Perspective

From an aquaculture perspective, this study determined the thermal tolerance limits and identified a more optimal intermediate thermal environment (18–20˚C), with efficient growth and fewer deformities, for future rearing of ELH stages of European eel. The next step would be to further optimize other environmental rearing conditions, such as salinity regime (similar to that used in Japanese eel culture) and combine this information with our existing knowledge (light and temperature). Together, these enhanced rearing conditions will provide a promising step towards the sustainable culture of this species. So far, the intermediate thermal optimum, identified here, is colder than the optimal thermal rearing conditions (25˚C) of the closely related Japanese eel [15]. Currently, the Japanese eel is the only eel species with a closed life cycle in captivity and the experimentally identified optimum thermal conditions are found to be similar to those encountered in their recently identified natural spawning area [15, 16]. In this regard, a recent study has shown that inducing vitellogenesis at 15˚C and final maturation at 18˚C, results in higher reproductive performance of European eel [68]. These results indicate that European eel reproductive maturation might occur in the deeper and colder layer while gamete development in a slightly warmer layer of the Sargasso Sea. Thus, the thermal tolerance limits identified in this study might not only advance the rearing conditions for future culture of European eel larvae but possibly also contribute to the understanding for further hypotheses regarding the natural spawning conditions and location. As such, the results of this study suggest that the habitat (or niche) of the earliest life stages of European eel in nature might be the characteristic "18˚C" ocean layer of the Sargasso Sea [7]. However, extrapolation of lab work to the field should always be approached carefully. Thus, the mystery of the exact location of European eel early life stages and their preferred conditions in nature still remains an enigma. However, we clearly show that increasing temperature had a deleterious impact on European eel embryonic and larval development and survival. Thus, our results support the recent hypotheses, that rising temperatures in the Sargasso Sea (linked to factors such as North Atlantic Oscillation and food availability) might show negative effects on eel recruitment [69–71].

Conclusion

In conclusion, this study provides important insights on phenotypic sensitivity to temperature and the underlying gene expression of the associated molecular mechanisms in European eel larvae. Temperature was found to influence all traits investigated, resulting in reduced larval stage duration at higher temperatures due to accelerated development, but with decreasing yolk utilization efficiency. The highest larval survival, combined with the lowest incidence of larval deformities at hatch and the highest yolk utilization efficiency that occurred when reared at 18˚C, as well as the correspondence with a high *gh* and low *hsp* expression, indicate a more optimal environment for early life development and rearing. Understanding the biological responses, limits and adaptabilities or preferences to extrinsic environmental factors, such as temperature, provides enhanced knowledge for the optimization of rearing techniques of a socially and economically important species such as European eel, as well as insights into its ecology.

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Study 2:

Temperature induced variation in gene expression of thyroid hormone receptors and deiodinases of European eel (*Anguilla anguilla*) larvae

Politis SN, Servili A, Mazurais D, Zambonino-Infante J-L, Miest JJ, Tomkiewicz J, Butts IAE

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Temperature induced variation in gene expression of thyroid hormone receptors and deiodinases of European eel (*Anguilla anguilla*) larvae

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ABSTRACT

Thyroid hormones (THs) are key regulators of growth, development, and metabolism in vertebrates and influence early life development of fish. TH is produced in the thyroid gland (or thyroid follicles) mainly as T4 (thyroxine), which is metabolized to T3 (3,5,3′-triiodothyronine) and T2 (3,5-diiodothyronine) by deiodinase (DIO) enzymes in peripheral tissues. The action of these hormones is mostly exerted by binding to a specific nuclear thyroid hormone receptor (THR). In this study, we i) cloned and characterized *thr* sequences, ii) investigated the expression pattern of the different subtypes of *thrs* and *dios*, and iii) studied how temperature affects the expression of those genes in artificially produced early life history stages of European eel (*Anguilla anguilla*), reared in different thermal regimes (16, 18, 20 and 22 °C) from hatch until first-feeding. We identified 2 subtypes of *thr* (*thrα* and *thrβ*) with 2 isoforms each (*thrαA*, *thrαB*, *thrβA*, *thrβB*) and 3 subtypes of deiodinases (*dio1*, *dio2*, *dio3*). All *thr* genes identified showed high similarity to the closely related Japanese eel (*Anguilla japonica*). We found that all genes investigated in this study were affected by larval age (in real time or at specific developmental stages), temperature, and/or their interaction. More specifically, the warmer the temperature the earlier the expression response of a specific target gene. In real time, the expression profiles appeared very similar and only shifted with temperature. In developmental time, gene expression of all genes differed across selected developmental stages, such as at hatch, during teeth formation or at first-feeding. Thus, we demonstrate that *thrs* and *dios* show sensitivity to temperature and are involved in and during early life development of European eel.

1. Introduction

The European eel (*Anguilla anguilla*) has been subjected to extensive scientific inquiry due to its enigmatic natural ecology (Van Ginneken and Maes, 2005; Miller et al., 2015; Righton et al., 2016), the critically endangered status of the population (Jacoby and Gollock, 2014), and the ongoing natural and anthropogenic pressures on the already historically low stock (Friedland et al., 2007; Bonhommeau et al., 2008; Geeraerts and Belpaire, 2010; ICES, 2015). As such, researchers are now employing technological means to circumvent the European eel decline and are striving to establish a sustainable aquaculture. Unfortunately, the aquaculture industry for European eel has been drastically reduced (ICES, 2015) due to the fact that it is capture-based, relying on wild-caught offspring (juvenile glass eels), which are unsustainably farmed until marketable size. The earliest life stages of European eel have not been encountered in nature, thus research needs to focus on laboratory studies to overcome current bottlenecks and gain knowledge about the species physiology and ecology. With recent advances in assisted reproduction (Butts et al., 2014; Müller et al., 2016; da Silva et al., 2016) and larval culture of European eel (Politis et al., 2014a; Sørensen et al., 2016; Butts et al., 2016) this is now possible and warrants further investigation. In addition, the European eel genome has recently been sequenced (Henkel et al., 2012), which offers new and emerging perspectives for fundamental molecular studies in eel biology (Rozenfeld et al., 2016).

In order to close the life cycle in captivity, similar bottlenecks needed to be overcome regarding the closely related Japanese eel (*Anguilla japonica*), which was accomplished in recent years (Masuda et al., 2012; Tanaka, 2015). Extensive efforts have been devoted to reach breakthrough achievements, forming the baseline

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knowledge for eel research (Tanaka et al., 2001, 2003). Subsequently, several studies have focused on assessing the extrinsic (environmental) and intrinsic (genetic) requirements and preferences of all life stages in order to identify the most optimal conditions for rearing early life history (ELH) stages of this species (Yamano et al., 2007; Ahn et al., 2012; Hsu et al., 2015). However, still there seems to be large variation and differences in larval growth of artificially and naturally reproducing eels (Miller, 2009). Environmental factors such as temperature have been shown to drastically influence larval morphology and survival (Okamura et al., 2007; Ahn et al., 2012). Moreover, it was shown that the thyroid hormone (TH) pathway may control the early life development of Japanese eels, similar to Japanese conger eels (*Conger myriaster*) through thyroid hormone receptors (THR) (Kawakami et al., 2003; Kawakami et al., 2013). Thus, TH treatment has been applied to enable synchronized metamorphosis leading to a lower developmental variability (Yamano et al., 2007).

Generally, THs are key regulators of growth, development, and metabolism in vertebrates (Power et al., 2001, Warner and Mittag, 2012, Tata, 2006). THs are produced in the thyroid gland (or thyroid follicles) mainly as T4 (thyroxine), which is metabolized to T3 (3,5,3′-triiodothyronine) and T2 (3,5-diiodothyronine) by deiodinase enzymes (DIO1-3) in peripheral tissues (Köhrle, 2000). The action of THs are mostly exerted by binding to specific nuclear THRs, although a non-genomic pathway has been shown to mediate TH functions through cellular signal transduction systems and cell surface receptors (Davis et al., 2008). THRs belong to the subfamily I of nuclear hormone receptors and share a modular structure composed of a N-terminal region (termed A/B domain), a conserved DNA binding domain (DBD or C region), a less conserved hinge region (D) followed by the ligand binding domain (LBD or E domain), and the C-terminal region (F region). In mammals, two genes (*thrα* and *thrβ*) generally encode THRα and THRβ (Lazar et al., 1993), whereas several genes encoding THRα and THRβ can be found in fish, due to teleost genome duplication events (Nelson and Habibi, 2009). For instance, two *thrα* genes have been described in zebrafish, *Danio rerio* (Takayama et al., 2008) and other fish species (Bertrand et al., 2007), while two *thrβ* have been reported in Japanese conger eel (Kawakami et al., 2003).

Increasing evidence suggests that THs have important roles during early life development and metamorphosis in fish (Marchand et al., 2004, Walpita et al., 2007, Infante et al., 2008, Campinho et al., 2010). The Japanese conger eel (Kawakami et al., 2003) and the Japanese eel (Kawakami et al., 2013) were previously chosen as model species to investigate the TH role on development and metamorphosis of Anguilliformes. An investigation of two isoforms of *thrα* (*thrαA* and *thrαB*) and two isoforms of *thrβ* (*thrβA* and *thrβB*) showed differentially regulated gene expression for all isoforms during development and metamorphosis in Japanese eel (Kawakami et al., 2013). Thyroid hormones induce differential transactivation activity of the different *thr* isoforms, and interestingly, elevated gene expression was observed between hatching and the transition to exogenous feeding (Kawakami et al., 2013). Thus, it is of importance to further elucidate the *thr* expression profiles during this sensitive period of anguillid ELH, but especially during the still mysterious ELH of European eel.

The objectives of this study were to i) clone and characterize *thr* sequences, ii) follow the expression pattern of *thrs* and *dios*, and iii) investigate how temperature affects the expression of those genes in artificially produced ELH stages of European eel, reared in different thermal regimes from hatch until first-feeding.

2. Materials and methods

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2012-15-2934-00458). Adult eels were anesthetized using ethyl p-aminobenzoate (benzocaine) before tagging and handling. Endogenously feeding larvae of European eel were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222). All efforts were made to minimize animal handling and stress.

2.1. Broodstock management and gamete production

Female silver eels were wild-caught (lake Vandet, Jutland, Denmark), while male eels were farmed (Stensgård Eel Farm A/S). Prior to experimental maturation, the fish were transported to a DTU Aqua research facility (Lyksvad Fishfarm, Vamdrup, Denmark), and housed in 300 L tanks equipped with a recirculation system (Tomkiewicz, 2012). Eels were maintained under low intensity light (~20 lux), 12 h day/12 h night photoperiod, salinity of ~36 ppt, and temperature of 20 °C. Acclimatization took place over 14 days and the eels were not fed during the entire experimental period. Furthermore, eels were anaesthetized (ethyl p-aminobenzoate, 20 mg L^{-1}; Sigma-Aldrich, Missouri, USA) and tagged with a passive integrated transponder. To induce vitellogenesis females received weekly injections of salmon pituitary extract (Argent Chemical Laboratories, Washington, USA) at 18.75 mg kg^{-1} body weight (Kagawa et al., 2005; Tomkiewicz, 2012). To stimulate follicular maturation and induce ovulation, females received 17α,20β-dihydroxy-4-pregnen-3-one (Sigma-Aldrich, Missouri, USA) at 2 mg kg^{-1} body weight (Ohta et al., 1996) and were strip-spawned after 12–14 h. Males received weekly injections of human chorionic gonadotropin (Sigma-Aldrich, Missouri, USA) at 150 IU/fish (Tomkiewicz, 2012). Prior to fertilization, an additional injection was given and milt was collected by strip-spawning ~12 h after administration of hormone. Milt samples were pipetted into a P1 immobilizing medium (Peñaranda et al., 2010) and only males with sperm motility of category IV (75–90%) were used for fertilization, within 4 h of collection (Butts et al., 2014). Eggs from each female were strip-spawned and fertilized separately by different sperm pools to create individual parental crosses. Subsequently, 0.2 µm filtered UV sterilized North Sea seawater was added for a gamete contact time of 5 min. Seawater was adjusted to 20 °C (±0.1 °C) and 36 (±0.1) ppt using Red Sea Salt (Red Sea Europe, Verneuil-sur-Avre, France). Only floating viable eggs/embryos were further used for experimentation.

2.2. Experimental conditions and design

During the spawning season, 25% of females successfully produced larvae after assisted reproduction treatments. Females represented here (n = 4) had a mean (±SEM) standard length and body weight of 65 ± 4 cm and 486 ± 90 g, respectively, while males (n = 11) had a mean (±SEM) standard length and body weight of 40 ± 3 cm and 135 ± 25 g, respectively. In this study, four individual parental crosses were used. Egg density for each parental combination was determined by counting 3 × 0.1 mL subsamples from the floating layer. Within 30 min post fertilization, ~500 floating viable eggs/embryos per 100 mL, with a mean size (±SD) of 1.5 ± 0.1 mm (at 2 h post fertilization), were distributed in replicated 600 mL flasks [182.5 cm^2 sterile tissue culture flasks with plug seal caps (VWR®)]. Seawater was supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA), which previously has been shown to increase survival in a controlled experimental environment (Sørensen et al., 2014). Embryos and larvae, from each parental cross, were reared in thermally con-

trolled incubators (MIR-154 Incubator, Panasonic Europe B.V.) at five temperatures (16, 18, 20, 22, and 24 °C ± 0.1 °C). All experimental units were acclimatized to the appropriate temperature regime within 1 h and salinity was kept at 36 ± 1 ppt. Temperature and salinity conditions were chosen to closely resemble the environmental conditions encountered at different depths of the assumed spawning areas in the Sargasso Sea (Castonguay and McCleave, 1987). Rearing of embryos and larvae took place in darkness, while handling and sampling was conducted under low intensity (<2.2 μmol m^{-2} s^{-1}) light conditions (Politis et al., 2014a). At hatch and every second day post-hatch until the corresponding first-feeding stage (Fig. 1), ~30 larvae from each parental cross (×4), each temperature (16, 18, 20 and 22 °C), and each replicate (×2) were randomly sampled for later molecular analyses. Those endogenously feeding larvae were immediately euthanized using MS-222 (Sigma-Aldrich, Missouri, USA), rinsed with deionized water, preserved in a RNA Stabilization Reagent, and kept at −20 °C following the procedure suggested by the supplier (Qiagen, Hilden, Germany). For further details on experimental design, rearing conditions, as well as larval morphology and development see Politis et al. (2017).

2.3. Data collection

2.3.1. Cloning and molecular characterization

cDNA sequences for *thrs* were predicted from genomic DNA sequences originating from the European eel genome assembly (WGS Project: AZBK01), available in NCBI resources (Henkel et al., 2012). Briefly, blastn requests were performed from Japanese eel cDNA *thr* sequences on the European eel WGS database to identify genomic contigs, including *thr* genes. European eel *thr* cDNA sequences were then deduced from contigs using GENSCAN (http://genes.mit.edu/GENSCAN.html) software (Burge et al., 1997). The predicted transcript sequences for each *thr* gene were confirmed by sequencing cDNAs from European eel RNA amplification. Cloning was achieved using TOPO TA cloning kit (Invitrogen) with pCR™II-TOPO® vector, according to the manufacturer's instructions. Primers sequences were as follows:

thrαA (F) 5′-GGGTCGCTGAGGAGACCT-3′,
thrαA (R) 5′-GGTGCTCAGACGTCCTGGT-3′,
thrαB (F) 5′-GCCCAACGACCCTAACCTAT-3′,
thrαB (R) 5′-GGATAGTAAATCAGGCCCTTAATC-3′,
thrβA (F) 5′-GACTCCGAGGCCCTAATTCT-3′,
thrβA (R) 5′-GACTCCGAGGCCCTAATTCT-3′,
thrβB (F) 5′-GAAGACTGAGCCCTGAGGTG-3′,
thrβB (R) 5′-TCACATGTACCCGCATTTGT-3′.

The cloned cDNA was sequenced with T7 and Sp6 primers by Beckman Coulter Genomics Inc (United Kindom). European eel *thr* cDNA sequences have been deposited to GenBank® with accession numbers found in Table 1.

2.3.2. Phylogenetic analysis

A phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) with amino acid *thr* vertebrate sequences. The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. For the analysis, 1000 bootstrap replicates were carried out. Phylogenetic analysis was conducted in MEGA6 (Tamura et al., 2013). The GenBank accession numbers of the sequences used can be found in the Supplementary material.

2.3.3. Gene expression

For total RNA extraction, the larval pool (~30 larvae) of each replicate was homogenized in 800 μl Tri-Reagent (Sigma-Aldrich, Missouri, USA). After obtaining the aqueous phase by incubation in 160 μl chloroform, RNA was extracted using the InviTrap® Spin tissue RNA MiniKit (STRATEC Biomedical AG, Berlin-Buch, Germany) following the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry (260/280 = 2.12 ± 0.16, 230/260 = 2.16 ± 0.16), using Nanodrop ND-1000 (Peqlab, Germany). From the resulting total RNA, 680 ng were transcribed using the Quanta qScript cDNA Synthesis Kit (Promega, Germany), according to the manufacturer's instructions, including an additional gDNA wipe out step prior to reverse transcription [Quanta PerfeCta DNase I Kit (Promega, Germany)].

The expression levels of target genes (thyroid hormone receptors: *thrαA, thrαB, thrβA, thrβB* and deiodinases*: dio1, dio2, dio3*) and reference genes (*ef1α, 18 s, 40 s*) were determined by quantitative real-time PCR (qRT-PCR) using specific primers. The *ef1α*, *18 s*, *40 s* genes were chosen as housekeeping genes since 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). Using primer 3 software v 0.4.0 (http://frodo.wi.mit.edu/primer3/) primers were designed i) for *thrαA*, *thrαB*, *thrβA* and *thrβB* based on cloned cDNA sequences, ii) for *ef1α* and *40 s* based on sequences available in Genbank nucleotide database, and iii) for *dio1*, *dio3* and *18 s* based on sequences available in the European eel transcriptome EeelBase 2.0 database (http://compgen.bio.unipd.it/eeelbase/), as indicated in Table 1. To design the primers of *dio2* a European eel predicted cDNA sequence was used and the size of the amplicon obtained by amplification was checked (http://www.zfgenomics.com/sub/eel). All primers were designed for an amplification size ranging from 75 to 200 nucleotides and the amplicons obtained were of the expected size (Table 1).

Expression of genes in each larval sample (4 parental crosses × 2 replicates × 4 temperatures) were analyzed in technical triplicates using the qPCR Biomark™ HD system (Fluidigm) based on 96.96 (GE chips) dynamic arrays (Miest et al., 2015). 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 μl cDNA per sample at 10 min at 95 °C; 14 cycles: 15 s 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 against the geometric mean of the three housekeeping genes and analyzed using the ΔΔ Ct method (Livak and Schmittgen, 2001). Coefficient of variation (CV) of triplicates was calculated and checked to be <0.04 (Hellemans et al., 2007). If CV was found to be >0.04, triplicates were checked for outliers and if possible duplicate measurements were used. If the use of duplicates was not possible (CV > 0.04) the whole data point was omitted from the analysis. Moreover, malfunctions on the chip and/or unsuccessful RNA extractions can result in expression failures, as was the case for some samples in this study.

2.4. Statistical analyses

All data were analyzed using SAS statistical software (version 9.1; SAS Institute Inc., Cary, North Carolina). Residuals were tested for normality using the Shapiro–Wilk test and homogeneity of variances was tested using a plot of residuals versus fit values (PROC GLOT, SAS Institute 2003). Data were $\log_{10}$ or arcsine

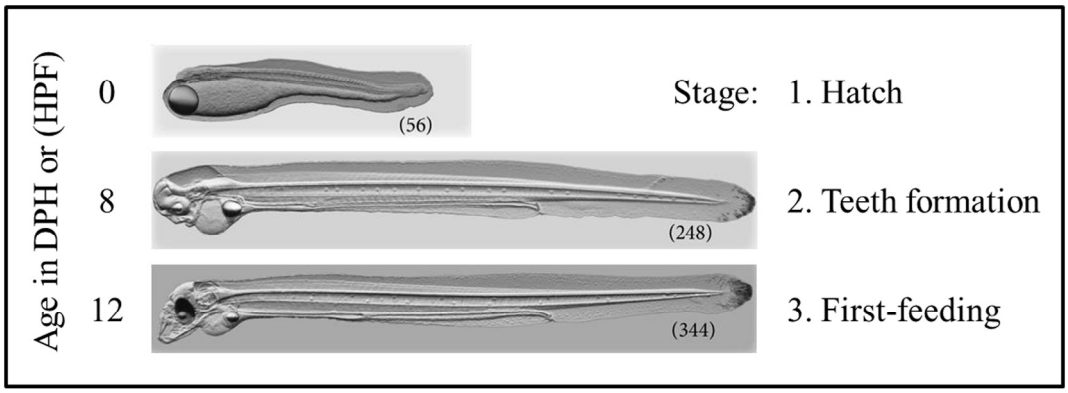

Fig. 1. Stage specific European eel larval development at 18 °C in days post hatch (DPH) and hours post fertilization (HPF). Stage 1 represents hatching, Stage 2 represents teeth formation, and Stage 3 represents first-feeding.

Table 1
Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR. Primers were designed from cloned THR cDNA sequences and other sequences available in GenBank Nucleotide, the European eel transcriptome database (EeelBase 2.0, http://compgen.bio.unipd.it/eeelbase/) or the eel genome website (http://www.zfgenomics.com/sub/eel). The table lists accession number and corresponding database of target gene sequences.

Full name	Abbreviation	Databases	Accession numbers	Primer sequence (5′-3′) (F: Forward; R: Reverse)
Deiodinase 1	dio1	EeelBase 2.0	Eeel2-c186	F: AGCTTTGCCAGAACGACTGT R: TTCCAGAACTCTTCGCACCT
Deiodinase 2	dio2	Eel genome website	g12347	F: GAAGAGGAGGATCGCCTACC R: GCACTCTACCTCCGTCCAAA
Deiodinase 3	dio3	EeelBase 2.0	Eeel-c22164	F: TACGGGGCGTATTTTGAGAG R: GCTATAACCCTCCGGACCTC
Thyroid Hormone Receptor alpha A	thrαA	GenBank Nucleotide	KY082904	F: GCAGTTCAACCTGGACGACT R: CCTGGCACTTCTCGATCTTC
Thyroid Hormone Receptor alpha B	thrαB	GenBank Nucleotide	KY082905	F: GAAGCCTTCAGCGAGTTCAC R: ACAGCCTTTCAGGAGGATGA
Thyroid Hormone Receptor beta A	thrβA	GenBank Nucleotide	KY082906	F: AGGAACCAATGCCAAGAATG R: GCCTGTTCTCCTCAATCAGC
Thyroid Hormone Receptor beta B	thrβB	GenBank Nucleotide	KY082907	F: GAAGACTGAGCCCTGAGGTG R: AGGTAATGCAGCGGTAATGG
Elongation Factor 1-alpha	ef1a	GenBank Nucleotide	EU407824.1	F: CTGAAGCCTGGTATGGTGGT R: CATGGTGCATTTCCACAGAC
Ribosomal 18S RNA	18 s	EeelBase 2.0	eeel2_s7245	F: AGAGCAGGGGAACTGACTGA R: ACCTGGCTGTATTTGCCATC
Ribosomal 40S RNA	40 s	GenBank TSA	GBXM01005349.1	F: TGACCGATGATGAGGTTGAG R: GTTTGTTGTCCAGACCGTTG

square-root-transformed when data deviated from normality and/or homoscedasticity (Zar, 1996).

Statistical models were used to investigate temperature effects on gene expression throughout early larval development [Ages 0–18 days post hatch (dph)] and at specific developmental stages (Stages 1–3). Across the different temperature treatments, Stage 1 represents the day of hatch, Stage 2 represents the timing of teeth formation, and Stage 3 represents the first-feeding stage (Fig. 1). Together, this allowed us to decipher changes in temperature at real-time and standardized developmental intervals.

To examine the effect of temperature on gene expression throughout early development (real time), we used two statistical approaches. In the first approach, we analyzed the data using a series of mixed-model repeated measures ANOVAs (PROC MIXED; SAS Institute 2003). Models contained the Temperature (16, 18, 20 and 22 °C) and Age (0–18 dph) main effects as well as the Temperature × Age interaction. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate (Littell et al., 1996). Temperature and Age were considered fixed, whereas parental cross was considered random. Tukey's post hoc analyses were used to compare least-squares means between treatments. If a significant Temperature × Age interaction was detected the model was decomposed into a series of reduced ANOVA models to determine the effect of Temperature for each Age and of Age for each Temperature. This was the case for *dio3* and *thrαA*. The reduced models involved only preplanned comparisons and did not include repeated use of the same data, so a-level corrections for a posteriori comparisons were not necessary.

In the second approach, we examined variation in gene expression, throughout development at each temperature, by fitting either linear, quadratic or cubic equations to the data (PROC REG; SAS Institute 2003). This allowed us to create predictive models to explore the shape of variation throughout development for each temperature. Linear, quadratic and cubic equations were chosen a priori to fit the data based on the available literature (e.g. McDonald, 2009; Sørensen et al., 2016). Final equation selection (linear, quadratic or cubic) was based on an F-statistic: $d.f._j \times (R_j^2 - R_i^2)/(1 - R_j^2)$, where: R_i^2 = the R^2 for the i-th order, R_j^2 = the R^2 for the next higher order, $d.f._j$ = the degrees of freedom for the higher-order equation with j degrees of freedom in the numerator and $d.f._j = n - j - 1$ degrees of freedom in the denominator (McDonald, 2009). Graphs and regressions were prepared in SigmaPlot (Version 13.0).

3. Results

3.1. Cloning and molecular characterization

In this study, two forms of THRα (termed aaTHRαA and aaTHRαB) as well as two forms of THRβ (named aaTHRβA and aaTHRβB) were cloned in European eel larvae. The full CDS (coding DNA sequence) of the four THRs was sequenced. The nucleotide sequences of aaTHRαA (GenBank accession number KY082904) and aaTHRαB (accession number KY082905) are 1375 bp and 1334 bp long, with an open reading frame (ORF) that codes for a protein of 416 and 435 amino acids respectively, while the nucleotide sequences of aaTHRβA (GenBank accession number KY082906) and THRβB (GenBank accession number KY082907) are 1288 bp and 1299 bp long, coding for 396 and 378 amino acid proteins respectively (see Supplementary material).

3.2. Phylogenetic analysis

The phylogenetic tree constructed by the Neighbor-Joining method from multiple sequence alignment of *thr* amino acid sequences from a range of vertebrates reveals that European eel *thrα* and *thrβ*, grouped into two clearly separated clades (99 bootstrap for both clades). In addition, the topology of the phylogenetic tree showed that European eel *thr* sequences clustered within the teleost group and each time the Japanese eel homolog *thr* sequence was the evolutionary closest (Fig. 2).

3.3. Gene expression

No larvae survived at 24 °C. Gene expression of selected genes was compared across the other temperature treatments (16, 18, 20 and 22 °C) in real time and at specific developmental stages (developmental time). The expression of target genes was affected in real and/or developmental time by larval age (or stage), temperature, and/or their interaction (see specific genes below).

3.3.1. Deiodinases

In developmental time, expression of *dio1* significantly (P = .004) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant (P = .271) effect of Temperature was detected (Fig. 3A and B). In real time, gene expression of *dio1* was significantly affected by Temperature (P = .034) and Age (P < .0001), while no significant Temperature × Age interaction was detected (P = .903). Gene expression of *dio1* significantly increased at 22 °C (Fig. 3C) and significantly increased throughout ontogeny with increasing larval Age (Fig. 3D). The relationship between Age and *dio1* expression was best explained by a sigmoidal cubic regression at 16 °C (Fig. 3E). There were no significant relationships between Age and *dio1* expression (Fig. 3F-H) at the other temperatures.

Similarly, expression of *dio2* significantly (P = .008) increased on Stage 3 (first-feeding), while no significant effect of Temperature was detected (P = .178; Fig. 3I and J) in developmental time. In real time, gene expression of *dio2* increased throughout ontogeny with increasing larval Age (P < .0001; Fig. 3L), while no significant Temperature (P = .088; Fig. 3 K) or Temperature × Age interaction (P = .394) was detected. The relationships between Age and *dio2* expression were best explained by sigmoidal cubic regressions at 16 °C, 18 °C and 20 °C (Fig. 3M-O). There was no significant relationship between Age and *dio2* expression at 22 °C (Fig. 3P).

On the other hand, expression of *dio3* significantly (P < .0001) increased on Stage 1 (hatch) and was significantly impacted by Temperature (P = .014; Fig. 3Q and R) when compared in developmental time, as well as significantly affected by the Temperature × Age interaction (P < .0001) when compared in real time. Thus, the model was decomposed into a series of reduced ANOVA models to determine the effect of Temperature for each Age (Fig. 3S) and of Age for each Temperature (Fig. 3 T-W). Significant differences in gene expression of *dio3* among temperatures occurred throughout development on 0 (P = .030), 2 (P = .019), 10 (P = .044), 12 (P < .0001) and 14 dph (P = .006; Fig. 3S). Larval Age significantly influenced gene expression of *dio3* when larvae were reared at 16, 18 and 20 °C (P < .0001; Fig. 3 T-V), but not at 22 °C (P = .056; Fig. 3 W). The relationships between Age and *dio3* expression can be explained by quadratic parabola regressions at all temperatures investigated (Fig. 3 T-W).

3.3.2. Thyroid hormone receptors

In developmental time, expression of *thrαA* significantly (P < .0001) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant (P = .167) effect of Temperature was detected (Fig. 4A and B). In real time, gene expression of *thrαA* was significantly affected by the Temperature × Age interaction (P = .039). Thus, the model was as above decomposed into a series of reduced ANOVA models to determine the effect of Temperature for each Age (Fig. 4C) and of Age for each Temperature (Fig. 4D-G). Significant differences in gene expression of *thrαA* among temperatures occurred throughout development on 2 (P = .033) and 8 dph (P = .016; Fig. 4C). Larval Age significantly influenced gene expression of *thrαA* and relationships between Age and *thrαA* expression can be explained by linear regressions at 16 °C, 20 °C and 22 °C (Fig. 4D, F and G), or by a sigmoidal cubic regression at 18 °C (Fig. 4E).

Similar to *thrαA*, expression of *thrαB* significantly (P < .0001) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant (P = .267) effected of Temperature was detected (Fig. 4H and I). In real time, gene expression of *thrαB* was significantly affected by Temperature (P = .006; Fig. 4 J) and Age (P < .0001; Fig. 4 K), while no significant Temperature × Age interaction (P = .147) was detected. Gene expression of *thrαB* significantly increased at 22 °C (Fig. 4J) and significantly increased (up to 5-fold) throughout ontogeny with increasing larval Age (Fig. 4K). The relationships between Age and *thrαB* expression were best explained by linear regressions at 16 °C, 20 °C and 22 °C (Fig. 4L, N and O), or by a sigmoidal cubic regression at 18 °C (Fig. 4M).

In contrast to all the other *thrs*, expression of *thrβA* significantly (P = .001) decreased from Stage 1 (hatch), while no significant (P = .098) effect of Temperature was detected (Fig. 5A and B). In real time, gene expression of *thrβA* was not significantly affected by Temperature (P = .342; Fig. 5C) and no Temperature × Age interaction (P = .940) was detected. On the contrary, a significant (P = .0003) effect of Age was detected (Fig. 5D). The relationships between Age and *thrβA* expression were best explained by linear regressions at 18 °C and 22 °C (Fig. 5F, H). No significant relationships between Age and *thrβA* expression were detected at 16 and 20 °C (Fig. 5E, G).

Similar to *thrαs*, expression of *thrβB* significantly (P < .0001) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant (P = .203) effect of Temperature was detected (Fig. 5I and J). In real time, gene expression of *thrβB* was significantly affected by Temperature (P = .012; Fig. 5K) and larval Age (P < .0001; Fig. 5L), while no significant Temperature × Age interaction (P = .296) was detected. Gene expression of *thrβB* significantly increased at 16 °C and 22 °C (Fig. 5K), while significantly increased (up to 6-fold) throughout ontogeny with increasing larval Age (Fig. 5L). The relationships between Age and *thrβB* expression were best explained by linear regressions at 16 °C and 22 °C (Fig. 5M, P), or by quadratic parabola regressions at 18 °C and 20 °C (Fig. 5N, O).

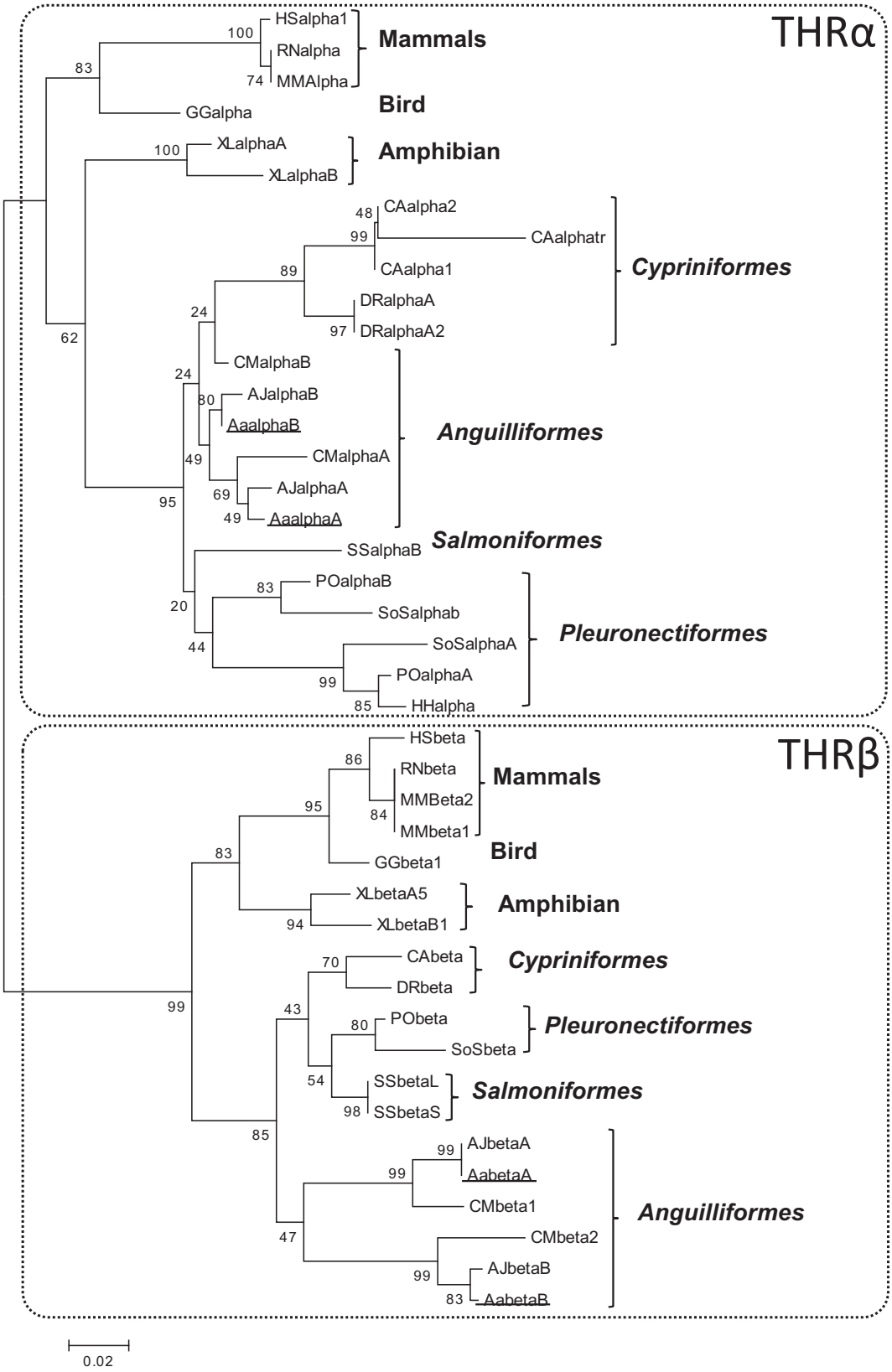

Fig. 2. Phylogenetic relationships among thyroid hormone receptors (THRs) of four vertebrate classes were inferred using the Neighbor-Joining method. Fish orders are in italics. The sequences of European eel, *Anguilla anguilla* obtained in this work are underlined. The percentages of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Phylogenetic analysis was conducted in MEGA6. S or L in the sequence's name stands for small and large protein forms. The mention tr indicates sequences of truncated proteins. Partial sequences have been omitted.

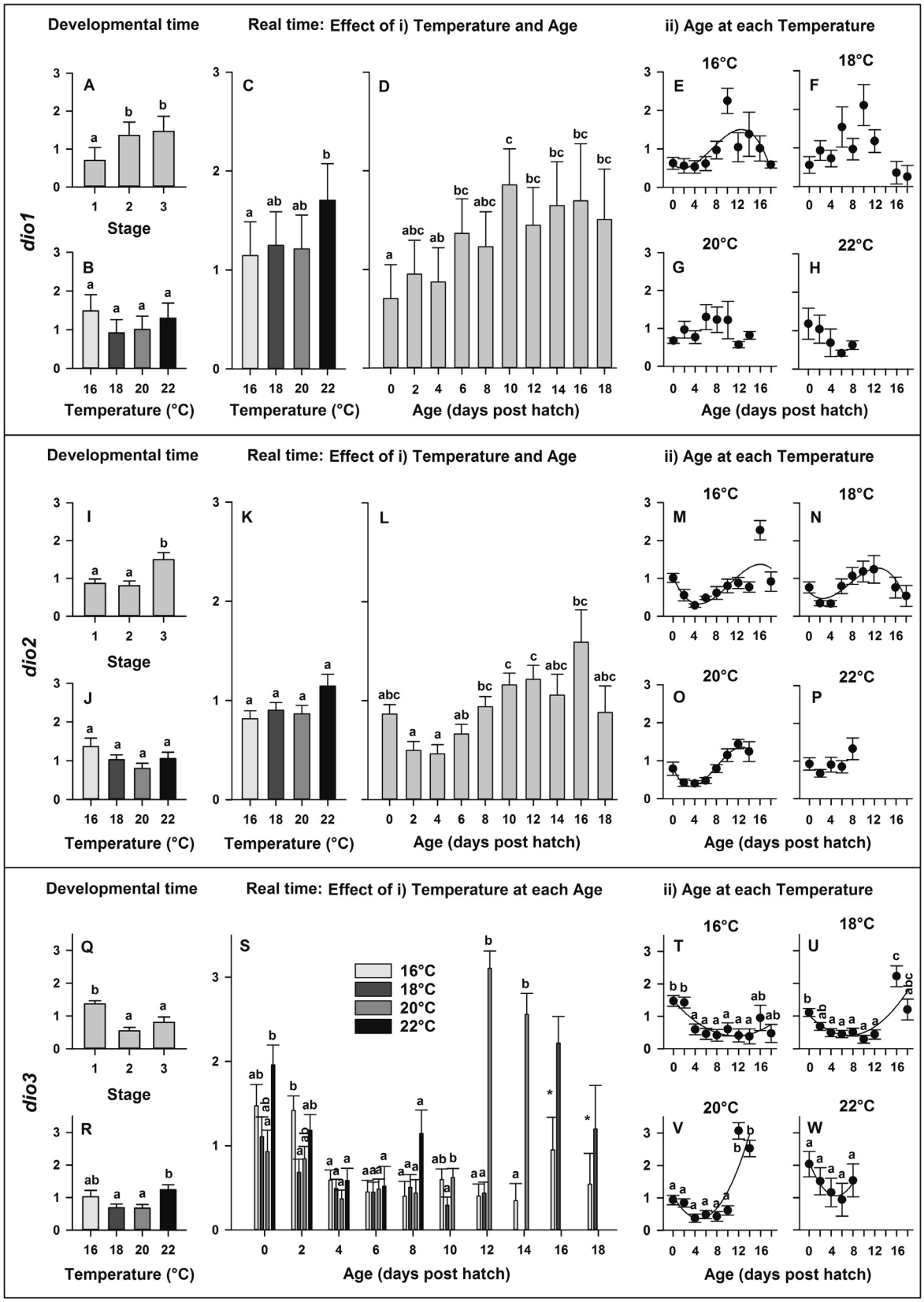

Fig. 3. Effect of temperature on larval European eel, *Anguilla anguilla* gene expression of *dio1* (A and B), *dio2* (I and J) or *dio3* (Q and R) at specific developmental Stages (1: hatch, 2: teeth formation and 3: first-feeding) and *dio1*(C and D), *dio2* (K and L) or *dio3* (S) in real time, as well as effect of age on *dio1* (E-H), *dio2* (M-P) or dio3 (T-W) expression at each temperature. Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (±SEM) among 4 crosses at each temperature. Means were contrasted using the Tukey-Kramer method and treatments with the same letters are not significantly different (P > .05).

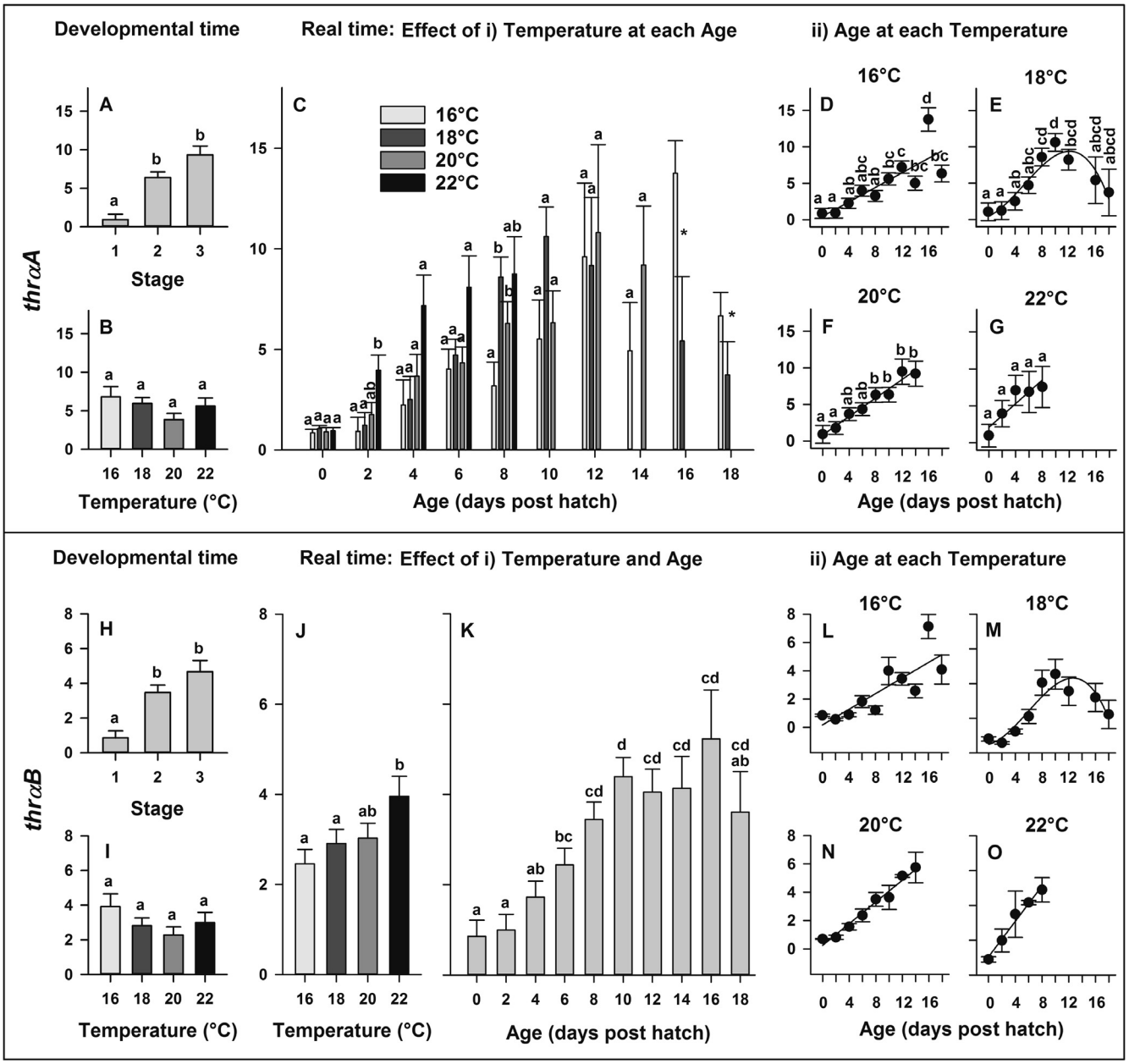

Fig. 4. Effect of temperature on larval European eel, *Anguilla anguilla* gene expression of *thrαA* (A and B) or *thrαB* (H and I) at specific developmental stages (1: hatch, 2: teeth formation and 3: first-feeding) and *thrαA* (C) or *thrαB* (J and K) in real time, as well as effect of age on *thrαA* (D-G) or *thrαB* (L-O) at each temperature. Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (±SEM) among 4 crosses at each temperature. Means were contrasted using the Tukey-Kramer method and treatments with the same letters are not significantly different (P > .05).

4. Discussion

This study aimed at enhancing our knowledge on the molecular mechanisms controlling European eel early life development. In fish, it has been suggested that THs play a role during the transitional period towards exogenous feeding and that *thr* expression can be affected by food deprivation (Raine et al., 2005). This study focused solely on the endogenous feeding period, where we investigated the expression patterns of genes encoding for THRs and deiodinases from hatching until first-feeding. Generally and within the thermal tolerance limits of each particular species, increasing temperature accelerates all biochemical processes, causing a shift in early fish development (Martell et al., 2005; Politis et al., 2014b) and gene expression can follow a similar pattern (Politis et al., 2017). In this study, we observed that the warmer the temperature the earlier the expression response of a specific target gene. Thus, even though the expression profiles often appeared very similar and only shifted with temperature (when compared in real time), they were affected (in real and/or developmental time) by larval age (or stage), temperature, and/or their interaction.

In more detail, fish ELH has been correlated to thyroid hormone metabolism, which is under the control of the hypothalamus-pituitary-thyroid axis, where the thyroid gland is synthesizing TH (Jarque and Piña, 2014). This is regulated by the thyroid stimulating hormone (thyrotropin), which is released by the pituitary as a consequence of the thyrotropin-releasing hormone from the hypothalamus (MacKenzie et al., 2009). TH is synthesized as a precursor (T4) with weak bioactivity and can be converted into active

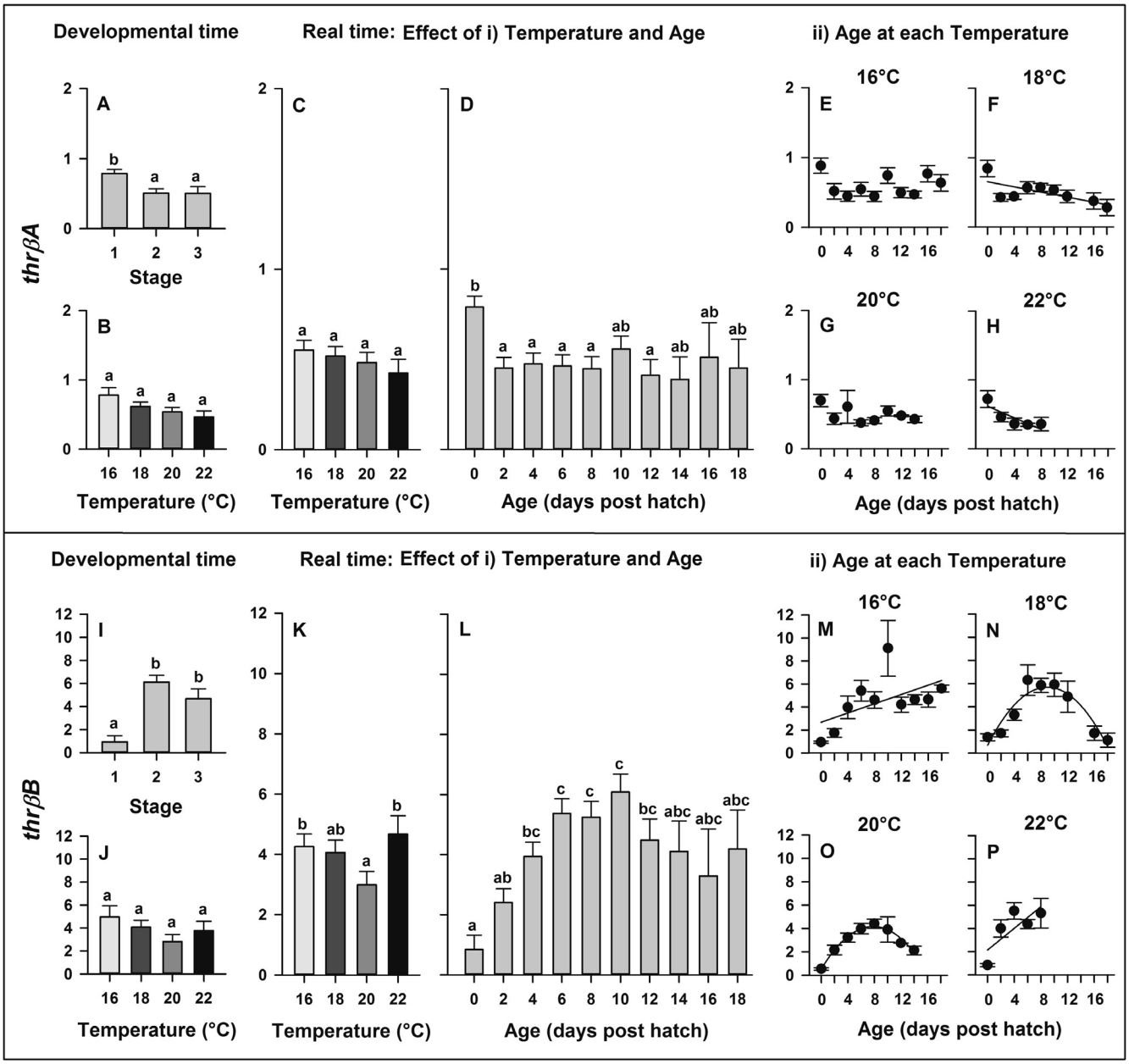

Fig. 5. Effect of temperature on larval European eel, *Anguilla anguilla* gene expression of *thrβA* (A and B) or *thrβB* (I and J) at specific developmental stages (1: hatch, 2: teeth formation and 3: first-feeding) and *thrβA* (C and D) or *thrβB* (K and L) in real time, as well as effect of age on *thrβA* (E-H) or *thrβB* expression (M-P) at each temperature. Values represent means (±SEM) among 4 crosses at each temperature. Means were contrasted using the Tukey-Kramer method and treatments with the same letters are not significantly different (P > .05).

hormones (T3) or inactive metabolites (reverse-T3 or T2) by three transmembrane proteins, the iodothyronine deiodinases (DIO1-3), in peripheral tissues. They selectively remove iodine moieties towards the active forms through the outer and the inactive forms through the inner ring deiodination processes (Jarque and Piña, 2014). Here, we *inter alia* identified and followed the expression patterns of all three iodothyronine deiodinase genes (*dio1-3*) during early (yolk-sac) larval stages of experimentally reared (from hatch until first-feeding) European eel. Interestingly, expressions of *dio2* and *dio3* were elevated at hatch, potentially due to maternal origin (Castillo et al., 1993; Olsen and Press, 1997; Huttenhuis et al., 2006; Swain and Nayak, 2009; Lee et al., 2013), or possibly representing their involvement in specific processes around hatching. Thus, it would be of interest to follow their expression patterns (and the consequences of potential variations) also during embryogenesis and embryonic development, in order to elucidate their role during this sensitive life period. Furthermore, the expression slowly decreased during the first couple of dph until a clear sigmoidal or exponential increase was observed, respectively. For *dio1*, we did not observe a similarly clear pattern in all thermal regimes.

Deiodination in the peripheral tissues of fish has been linked to several functional and vital processes, while showing sensitivity to environmental organic contaminants (Adams et al., 2000; Couderc et al., 2016), as well as physical environmental factors, such as pH (Mol et al., 1998), temperature (Adams et al., 2000), salinity (Orozco et al., 2002), and/or light (Comeau et al., 2000; Wambiji et al., 2011). Temperature has been shown to influence the outer and/or the inner ring deiodination of species, such as rainbow trout, *Oncorhynchus mykiss* (Johnston and Eales, 1995), blue tilapia,

Oreochromis Aureus (Mol et al., 1997), Atlantic cod, *Gadus morhua* (Cyr et al., 1998; Comeau et al., 2000), American plaice, *Hippoglossoides platessoides* (Adams et al., 2000) and zebrafish (Little et al., 2013). In the present study, we experimentally showed that temperature had a significant effect (in real and/or developmental time) on gene expression related to deiodination activities during early larval development of European eel. Moreover, we showed that the expression of all deiodination genes are significantly affected by the age or stage of eel larvae. Within the thermal tolerance limits, we commonly observed elevated expression towards and within the first-feeding window, which probably corresponds to a timing of refinement for organogenesis (e.g. brain, liver, gastro intestinal tract, etc.) and/or specific functional tissue (e.g. teeth, eyes, etc.), in order to ensure optimal transition from endogenous to exogenous feeding (Sørensen et al., 2016; Butts et al., 2016).

Thyroid hormones are moreover known to regulate the level of THRs, which are members of the steroid hormone receptor superfamily (Evans, 1988). Two subtypes, with isoforms within the subtypes, are found to be products of distinct genes or splice variants of the same gene (Kawakami et al., 2013). In the present work, we have identified and cloned the full CDS of two *thrα* (*thrαA* and *thrαB*) and two *thrβ* (*thrβA* and *thrβB*) gene forms in European eel larvae and followed their expression patterns from hatch to the first-feeding stage. All four European eel sequences contain the conserved DBD and LBD domains and two putative zinc fingers, providing evidence that they should be functional (see Supplementary material). The phylogenetic tree constructed, using several THR vertebrate sequences, reveals that European eel *thrs* are close to their fish orthologs, showing the highest homology (97–99%) with Japanese eel sequences (Kawakami et al., 2013). In *thrαB*, however, an insertion of 22 amino acids was observed at the N-terminus part (A/B domain), which is missing in the corresponding Japanese eel sequence.

Similarly, to the genes involved in the deiodination process, *thrs* were affected by both, larval age, and temperature. Expression of *thr* genes increased during development, usually in a linear or an exponential fashion, with the exception of *thrβA* that decreased during development. Similar to the expression of deiodinases, expression of *thrs* was usually high around the first-feeding stages, probably representing the involvement of the THRs towards and during the refinement of organogenesis and specific tissue for an optimal transition to exogenous feeding. Moreover, in real time, temperature was found to impact the expression pattern of *thr* genes. Generally, increasing temperature caused acceleration in development (Politis et al., 2017) and the warmer the temperature, the earlier the expression response of a specific target gene, potentially corresponding to the timing of specific ontogenic development. Interestingly, the expression of *thrβA* showed a slight increase at the first sampling points (hatch), which could theoretically be due to maternal origin (Castillo et al., 1993; Olsen and Press, 1997; Huttenhuis et al., 2006; Swain and Nayak, 2009; Lee et al., 2013), while it remained low during the rest of the investigated developmental period. This could imply that *thrβA* genes are not involved in ontogenic development of the ELH stages but potentially gain functionality during the later and/or adult stages of this species, as it has been presented for the related Japanese conger eel (Kawakami et al., 2003) and Japanese eel (Kawakami et al., 2013).

In conclusion, we cloned and characterized *thr* sequences, followed the expression pattern of *thrs* and *dios*, and showed that temperature affected the expression of those genes in artificially produced ELH stages of European eel, reared in different thermal regimes from hatch until first-feeding. We identified 2 subtypes of *thr* (*thrα* and *thrβ*) with 2 isoforms each (*thrαA*, *thrαB*, *thrβA* and *thrβB*) and 3 subtypes of deiodinases (*dio1*, *dio2* and *dio3*). All *thr* genes identified, show high similarity to other mammalian, bird, amphibian or fish species, with highest similarity to the closely related Japanese eel. We can confidently state that all genes investigated in this study, involved in the mediation of TH action, were significantly affected (in real and/or developmental time) by larval age, temperature, and/or their interaction. Finally, we demonstrate that *thrs* and *dios* show sensitivity to temperature and are involved in and during early life development of European eel.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2017.11.003.

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Study 3:

Larval fish immunity at variable temperatures

MiestJJ, Politis SN, Tomkiewicz J, Butts IAE

Fish and Shellfish Immunology

in review

Larval Fish Immunity at Variable Temperatures

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Abstract

Temperature is a major factor that modulates the development of the immune system and its reactivity. Limited knowledge exists on the immune system of fish larvae, especially during the early life history stages. This study experimentally investigated the molecular ontogeny of the immune system and the influence of temperature on this process in European eel, *Anguilla anguilla* larvae. Larvae were reared at four temperatures, spanning their thermal tolerance range (16, 18, 20, 22°C) and expression patterns of 11 immune genes were analysed throughout development, from hatch to 18 days post hatch (dph). At the larvae's optimum temperature (18°C) the pattern of immune gene expression revealed an immunocompromised phase between hatch and teeth-development (0-8 dph), caused by a lag period between initial protection and development of inherent immune competence. Additionally, at the lower end of the thermal spectrum (16°C) immune competency appeared reduced, whilst close to the upper thermal limit (22°C) larvae showed signs of thermal stress. Thus, protection against pathogens is probably impaired at temperatures close to the upper and lower critical thermal limit, impacting survival and productivity in hatcheries as well as recruitment in natural environments.

Key words: European eel, *Anguilla anguilla*, Gene Expression; Aquaculture; Climate Change

Introduction

With more than 34,000 species, teleost fish represent the largest class of vertebrates [1]. This diversity allowed them to adapt to every aquatic environment. However, in almost all species natural mortality is highest during early life and survival rates may be only a few percent [2]. Thus, subtle differences in survivorship can cause large differences in annual offspring production [3]. This is especially important for species that spawn only once in a lifetime, such as the European eel (*Anguilla anguilla*), as survival during early-life represents a substantial component of variation in lifetime fitness. An increased understanding of the physical and biological factors that influence mortality rates during these 'critical' developmental stages can enable aquaculture hatchery production, enhance recruitment predictions for fisheries, and aid in the conservation of this critically endangered species [4].

Teleost fish was the first phylogenetic group of organisms to develop an immune system that possesses both the innate and adaptive arm of the immune response, characteristic to higher vertebrates [5]. However, evidence has accumulated that newly hatched fish larvae are particularly sensitive to pathogens as their immune system is not fully developed during the first weeks of life [6]. Marine fish larvae are particularly vulnerable to pathogen-induced mortality as it can take up to three months until their immune response is fully functional [7]. During this time, the larvae solely rely on the innate arm of the immune system, whilst exposure to pathogens intensifies due to hatching, mouth opening, and first-feeding [8]. Knowledge of the development of the immune system is hence needed to design preventative methods against pathogen associated losses in aquaculture hatcheries and to better understand immune responses in variable aquatic ecosystems.

During development organisms are influenced by extrinsic (e.g. temperature) or intrinsic factors (such as genetic makeup) and their associated interactions [9,10]. Temperature is one of the main factors influencing marine ecosystems, as it defines the geographical distribution of populations and affects the physiology of individual organisms at all life stages [11]. Furthermore, physiological processes, and therefore development and survival, in ectothermic organisms are generally controlled by the environmental temperature [12]. Additionally, early life history stages are known to be most sensitive to temperature as they have a narrower thermal tolerance window than juveniles or adults and are thus more profoundly affected by even minor temperature changes and short heatwaves [13].

Moreover, temperature is a fundamental modulator of the immune system of fish [14] and has been shown to affect immunity during fish early life history [15]. The consideration of temperature as an immunomodulatory factor is therefore not only important in the development of hatchery technology of the European eel in order to optimise rearing protocols, but also in the light of environmental changes in the natural habitat of the early life history stages of this species. Here, warming temperatures of the ocean may influence the recruitment of the critically endangered European eel [16,17].

Eels, i.e. Anguilliformes, are basal bony fish (Teleostei) which belong to the ancient superorder of Elopomorpha. This superorder is at the phylogenetic basis of Teleostei [18] and whilst the immune system of fish is well studied in some model species, very little research has been conducted regarding the immune system of Elopomorpha with their unique leptocephalus

larvae. The current knowledge of eel immunity has been reviewed by Nielsen and colleagues [19]. Research conducted on the immune system of European and Japanese eels (*A. japonica*) has up to date focused on the cellular innate immune response to infections and have rarely involved molecular studies [19]. For example, in Japanese eel it has been shown that some immune factors (i.e. lectin) are present 8 days post hatch (dph; rearing temperature unknown), whilst the appearance of most immune organs occurs later during larval development [20].

The European eel is a commercially important fish species with a long tradition in European fisheries and fish farming. However, up-to-date farming and restocking of European eel relies on wild-caught juveniles as the life-cycle has not been closed in captivity. As recruitment and stock size of European eel have decreased substantially in the last decades [21], the urge for captive production of offspring is ever increasing. It is therefore vital that breeding-technologies and hatchery techniques are being established. Recent advances have enabled the stable production of eggs and larvae, which allow the development and optimisation of hatchery protocols [22,23].

This study aimed to i) shed light on the ontogeny of the immune response during early European eel larval development; and ii) investigate the interaction of immune system related gene expression with temperature during early life history. These insights may be critical to close the life cycle of this commercially important fish species for aquaculture, and to better understand potential impacts of ocean warming on early life stages in nature.

Results

Generally, increasing temperature accelerated development, resulting in larvae reaching the first-feeding stage in 8 days at 22°C, 10 days at 20°C, 12 days at 18°C, and 16 days at 16°C [24]. For details on the observed regressions and Δct-values please refer to Table S2 and S3 in the supplementary material. If not otherwise indicated, x-fold values are given as mean ± SEM of all temperatures combined.

Two of the tested genes (complement component *c3* and immunoglobulin M (*igm*)) experienced a reduction in expression after stage 1 (hatch; $P < 0.0001$; Fig. 1 a and i) and a similar decreasing effect with age was observed in real time ($P < 0.0001$; Fig. 1 d and l). Interestingly, the other tested component of the complement system, *c1qc* and the T-cell marker *cd3* were not detected in the larvae until the end of the experiment. A member of the adaptive immune system investigated was major histocompatibility complex II (*mhc2*) and its expression, being upregulated beyond stage 2 (teeth formation; $P = 0.001$; Fig. 1 q), contrasted that of *igm* and *cd3*. Additionally, this gene was significantly affected by the rearing temperature since expression increased more than 2-fold at 16 and 22°C ($P = 0.003$) compared to the other temperatures (Fig. 1 r). The real-time age analysis revealed a significant temperature × age interaction ($P = 0.043$) leading to a ~13-fold increase in expression on 4 and 6 dph and 40-fold on 8 dph at 22°C compared to the colder temperatures (all $P \leq 0.01$; Fig. 1 s). Furthermore, larval age significantly influenced expression of *mhc2* when larvae were reared at temperatures ranging from 16 to 20°C ($P \leq 0.01$), which led to a steady increase in mRNA levels throughout larval development (Fig. 1 t-w).

As shown in Fig. 1 x, expression of the pathogen recognition receptor *tlr2* (toll like receptor 2) approximately doubled at stage 2 (teeth formation) and tripled at stage 3 (first-feeding) ($P <$

0.0001). Similarly, expression of *tlr2* was also significantly affected in real time ($P < 0.0001$), where its expression significantly increased with increasing age from a minimum expression of 0.62 ± 0.09-fold on 2 dph to a maximum expression of 4.34 ± 0.23-fold on 16 dph (Fig. 1aa).

Interferon regulating factors (*irf*) 3 and 7 increased by 2 - 3-fold beyond stage 2 (teeth formation) ($P < 0.0001$; Fig. 2 a and i). In real-time, expression of *irf3* was significantly affected by temperature ($P = 0.029$) and larval age ($P < 0.0001$), though no significant temperature × age interaction was detected. Gene expression of *irf3* was significantly elevated (6.66 ± 0.69-fold) at 22°C compared to 4.59 ± 0.45-fold at 16°C (Fig. 2 c) and significantly increased throughout ontogeny with increasing larval age (Fig. 2 d). Here, expression increased in a linear manner from 2.24 ± 0.25-fold on 0 dph to reach a maximum of 8.26 ± 0.78-fold on 16 dph. On the contrary, *irf7* was significantly affected by the temperature × age interaction ($P = 0.013$). Significant differences in mRNA levels of *irf7* among temperatures occurred on 4 dph ($P = 0.045$), where expression at 22°C was about 40% higher than the expression at 16°C (Fig. 2 k). Additionally, *irf7* levels steadily increased throughout development in all temperatures (16-22°C, $P \leq 0.007$, Fig. 2 l-o).

Moreover, two pro-inflammatory cytokines were investigated. Gene expression of tumor necrosis factor α (*tnfα*) was low (ct > 26) throughout the studied time period and was not affected by temperature and larval age (or stage), and no temperature × age (or stage) interaction was detected (data not shown). Interleukin 1β (*il1β*) on the other hand, was only significantly increased at stage 3 (first-feeding) ($P = 0.033$) by approx. 3-fold (Fig. 2 p). The real time analysis however, revealed a significant temperature × age interaction ($P = 0.003$). Significant differences in gene expression of *il1β* among temperatures occurred on 6, 8 and 12 dph ($P < 0.02$; Fig. 2 r). On 8 dph in specific, expression levels of *il1β* were approximately 6-fold higher at 22°C compared to the other temperatures. Additionally, a significant increase in *il1β* expression with increasing age was observed at all rearing temperatures ($P \leq 0.001$) except at 20°C (Fig. 2 s-v), probably due to high levels of variation between individuals at this temperature.

In developmental time, the only other gene besides *mhc2* affected by temperature was the antimicrobial enzyme c-type lysozyme (*lysc*, Fig. 2 x) and *lysc* expression steadily increased throughout development from hatch to the first-feeding stage (Fig. 2 w). Moreover, in real time the 22°C treatment caused a strong (300%) upregulation of this gene on 8 dph compared to the other temperatures (Fig. 2 y), while the 16°C treatment on the other hand led to a significant lower *lysc* expression on 12 and 14 dph (Fig. 4 y). Additionally, a significant ($P \leq 0.001$) increase in *lysc* expression with increasing age was observed at all rearing temperatures (Fig. 2 z-ac).

Discussion

Fish larvae are particularly sensitive to pathogens as their immune system is not fully developed [7]. By studying the timing of expression of several immune genes during development from hatch to the first-feeding stage, we were able to identify a sensitive period, which is closely linked to survival (conceptualized in Fig. 3). This sensitive period is caused by a lag period between depletion of initial protection and build-up of the larvae's inherent immune system. Initial protection at hatch was exerted by complement component C3 and immunoglobulin Type M. These two factors, which might have been transferred maternally [34–38], work together to facilitate

binding of opsonized bacteria to complement receptors on phagocytes [37,39]. During regular ontogeny at the larvae's optimal temperature (18 - 20°C, [24]), *c3* and *igm* were highly expressed at hatch. Their depletion was closely linked to the shrinking of the yolk sac area [24] leading to baseline levels after approximately 2 dph. A similar pattern had been previously observed in larvae of other species [40,41] and could thus indicate a more general pattern in fish larvae. In contrast, the other innate and adaptive immune factors tested revealed a slow but steady build-up of protection. For most genes tested, expression surpassed initial levels around 4 to 6 dph leaving the larvae in an immunocompromised state between approximately 2 and 6 dph. During this period larvae seemed to have already been able to recognise bacterial, parasitic, and/or viral pathogens, as this is indicated by the presence of mRNA of *tlr2*, *irf3* and *irf7* at hatch [42]. Additionally, a study in zebrafish showed that *tlr2* is already expressed before hatch [43]. Pathogen recognition is an integral part of the initiation of an innate and adaptive immune response and they play an important part in the immune surveillance throughout the different life stages [6]. Once a pathogen has been detected, it can be phagocytosed and eliminated intracellularly. One mechanism involved is the exposure of the pathogen to lysozyme. This antibacterial enzyme is one of the maternal immune factors that are transferred to the egg and it has been detected in the early life stages (oocytes to larvae) of various fish species [44–46]. This, together with the observed *tlr2* expression and the presence of skin lectin at 8 dph in Japanese eel, as demonstrated by Suzuki and Otake [20], indicates that innate antibacterial protection is an integral part in the immunological protection during early larval development in eel.

The innate immune response is conveyed through signalling molecules, such as the cytokines TNF-α and IL-1β, which initiate a cell signalling cascade leading to an inflammatory response upon pathogen recognition [47]. During eel ontogeny, expression of *il1β* increased steadily, indicating an increase in the potential to mount a pro-inflammatory immune response and also the presence of cytokine producing immune cells, such as macrophages. Another indicator for the presence of these cells is the increasing expression of *mhc2* throughout eel larval development. On the contrary, *tnfa* was not expressed during the first 18 dph. This is very dissimilar to patterns observed in the freshwater larvae of brown trout (*S. trutta*) and rohu (*L. rohita*) [46,48] and could be due to the delayed development of marine fish larvae [7]. Moreover, this is an indicator of an immature immune system at this early life stage in *A. anguilla*. Interestingly, studies of Japanese eel demonstrated that whilst *igm* and T-cell-related *lck* genes were expressed already 3 days post fertilisation, the development of lymphoid tissues were delayed and neither spleen, thymus nor lymphocytes were observed in larvae of similar sizes [20,49]. This is in line with the observed absence of *cd3* expression in the present study and it can therefore be assumed that adaptive immune protection is still underdeveloped in very early larval stages (i.e. pre-leptocephalus stage). As this would have implications for the use of vaccines during early life stages, further studies are needed to link the present findings to the functionality of the adaptive immune system.

A previous study [26] indicated an S-shaped survival curve in non-feeding European eel larvae; i.e. low initial mortality, followed by a steep decline in survival until mortality subsides. Similar patterns have also been observed in various fish species such as turbot [29], winter flounder [50], Pacific herring [51], and cod [52] larvae. A period of unusually high mortality during fish larval development has previously been proposed in "Hjort's critical period hypothesis", which

directly influences recruitment [53]. Previous studies link this period of high mortality to feeding success, size, predation, and life history strategy [53–55]. However, since such critical periods can also be observed in culture we hypothesized that they are also linked to pathogen sensitivity and that eel larvae are immunocompromised during early development. Our results indicate a sensitive phase during which larvae are immunocompromised and hence highly susceptible to pathogens (Fig. 3). The same figure also highlights that this sensitive phase closely reflects the survival rates of the larvae with an initial protection during the first two days, a steep increase in mortality rates until 8 dph, followed by a decelerated decline in survival once immunocompetence increases. The onset of the latter seems to coincide with the formation of the teeth. Thus, survival seems to be closely linked to immune ontogeny, which is not surprising as it is well established that during development innate immunity provides quick protection against the hostile environment [6]. Obviously, the study of gene expression can only give an indication of functionality and hence these findings should be confirmed in future through studies of immune organ development and pathogen challenge experiments. In order to increase survival rates and prevent high and unpredictable mortalities in aquaculture settings, it should thus be investigated if and how the onset of the innate immune system can be shifted earlier towards the time of hatch. Possible mechanisms would include maternal and larval immunostimulation as well as steering larval microbiota [29,56,57].

Moreover, we expected that environmental factors such as temperature would influence the demonstrated development of this essential defence system. Temperature is an important factor influencing aquatic life and oviparous fish are directly exposed to it already as gametes. This study therefore did not only aim to elucidate some aspects of the European eel's development of the immune system but also how this is influenced by the ambient temperature. Together, this can provide insights regarding European eel larval physiology, compulsory to defining optimal rearing conditions in culture as well as identifying potential effects of global warming on recruitment in nature.

In the present study, immune gene expression was studied until larvae reached the first-feeding stage. Temperature influences development and thus the age at which the larvae reach the first-feeding stage, i.e. 16 dph at 16°C, 12 dph at 18°C, 10 dph at 20°C and 8 dph at 22°C [24]. At the lower end of the thermal spectrum (16°C) immune defences appeared to be impaired as development is delayed and immune protein activity is reduced [58]. Generally, the genes investigated here, were robust to the environmental temperature but close to the critical thermal maximum (CTmax) a stress response was previously detected [24], which could therefore be linked to impaired pathogen protection. Moreover, mRNA levels of *c3* reflected a temperature dependent decrease of yolk-sac area (described in [24]). A temperature effect on initial protection especially during temperatures close to the critical thermal maximum (i.e. $CT_{max} \geq 22°$) can therefore not be excluded. Additionally, the present study did not investigate temperature effects on embryonic development and thus it cannot be excluded that temperature exposure prior to hatch would influence initial defence systems. Interestingly c-type lysozyme and *mhc2* levels were increased at the warmer end of the thermal window. Both genes experienced an overshoot when reaching the first feeding stage at 22°C, which is close to the upper thermal limit of these larvae [24]. This correlates with an increased expression of heat shock proteins 70 and 90 in the same larvae [24] and is an indication of temperature induced cellular stress and an immune response towards damaged

cells [59]. Protection against pathogens is thus probably impaired at temperatures close to CT_{max}, which in culture under suboptimal rearing schemes can be crucial to survival while in nature heat waves and rising sea surface temperatures could increase larval mortality and hence glass eel recruitment.

In conclusion, our study highlighted the influence of immune gene expression on larval survival in European eel. We identified an immunocompromised phase during which mortality is high and larvae are more vulnerable to pathogen infection. We are confident that future research will identify this as a more general effect in marine fish larvae. In addition, we were able to demonstrate the influence of temperature on larval immune gene expression close to their upper and lower thermal limit. These findings have important implications on rearing conditions and disease prevention protocols (e.g. timing of vaccination, immunostimulation treatments) of *A. anguilla* in culture and on our understanding of ocean warming impacts on recruitment.

Methods

Broodstock management and offspring production

Broodstock management and offspring production have been previously described in more detail [24]. Female silver eels were obtained from a freshwater lake, Vandet, Jutland, Denmark. Male eels were obtained from Stensgård Eel Farm A/S. Females used for experiments (n = 4) had a mean (± SEM) standard length and body weight of 65 ± 4 cm and 486 ± 90 g, respectively. Male eels (n = 11) had a mean (± SEM) standard length and body weight of 40 ± 3 cm and 135 ± 25 g, respectively.

Ethical approval

The experimental protocol for the study was approved by the Animal Experiments Inspectorate, Danish Ministry of Food, Agriculture and Fisheries (J.no. 2012-15-2934-00458). All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

Experimental conditions and sample collection

The experiments took place at a research facility of the Technical University of Denmark in Jutland, Denmark. Eggs from each female were "crossed" with a standardized milt pool of several males [25] to experimentally create four parental crosses. Within 30 min post fertilization, ~500 floating viable eggs/embryos per 100 mL, with a mean size (± SD) of 1.5 ± 0.1 mm (n = 4 females), were distributed in replicated 600 mL flasks [182.5 cm^2 sterile tissue culture flasks with plug seal caps (VWR®)]. Larvae were reared in thermal controlling incubators (MIR-154 Incubator, Panasonic Europe B.V.) at five temperatures (16, 18, 20, 22, and 24 ± 0.1°C), with a salinity of 36 psu. Seawater was 0.2 μm filtered, UV sterilized and supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) [26]. Rearing of embryos and larvae took place in

darkness, while handling and sampling was performed under low intensity light conditions (< 2.2 μmol m^{-2} s^{-1}) [27]. Salinity levels and the temperature range were chosen to resemble the environmental conditions prevailing between 0 and 600 m's depth in the Sargasso Sea, i.e. the assumed spawning area of the European eel.

For molecular analysis, ~30 larvae (× 2 replicates) from each temperature and parental combination were randomly sampled at hatch and every second day post-hatch until the corresponding first-feeding stage. As feeding trials were beyond the scope of this study, rearing was not conducted beyond these time points. Larvae were euthanized, using an aqueous solution of tricaine methane sulphonate (MS-222, Sigma-Aldrich, Germany), rinsed with deionized water, preserved in RNA-later (Qiagen, Germany), and kept at -20°C. No larvae hatched at 24°C and therefore this treatment was excluded from analysis.

Molecular analyses

This study took advantage of the assembly of the European eel genome [28] and obtained sequences were checked for high similarity with other fish species. Primers were designed for real-time PCR with Primer3plus (http://primer3plus.com/) and immune function of genes was verified.

Molecular analysis was performed at GEOMAR, Helmholtz Centre for Ocean Research in Kiel, Germany. Total RNA was extracted using a combination of Tri-Reagent (Sigma-Aldrich, Germany) and the InviTrap® Spin tissue RNA MiniKit (Stratec) following the manufacturer's instructions. RNA concentration was determined by Nanodrop ND-1000 (Peqlab, Germany) and normalized to a common concentration of 100 ng μl^{-1} with HPLC water. Consequently, 680 ng RNA were transcribed with the Quanta qScript cDNA Synthesis Kit (QuantaBio, Germany) as described by the manufacturer including a genomic DNA wipe-out step [Quanta PerfeCta DNase I Kit (QuantaBio, Germany)]. Controls for gDNA efficiency were also included and cDNA was stored at -20°C until further use.

From all larval samples (4 crosses × 4 temperatures × 2 replicates), the expression of 15 genes (*18s*, *40s*, *tubβ*, *ef1*, *c3*, *c1qc*, *cd3*, *igm*, *irf3*, *irf7*, *il1β*, *lysc*, *mhc2*, *tnfα*, *tlr2*; Table S1) was analysed in technical triplicates using the qPCR BiomarkTM HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips), as previously described [29]. Moreover, qBase+ software verified stability of housekeeping gene expression throughout analysed samples (M < 0.4; according to [30]). Gene expression was normalised (ΔCt) to the geometric mean of the three most stable housekeeping genes (*18s*, *40s*, *ef1*). Housekeeping (HK) gene expression was stable (M < 0.4) throughout the experiment (see Fig. S1, supplement) and variance in HK expression was clearly coupled to variation in cDNA amount in samples. Analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method, in relation to the 16°C sample of Day 0 from female 1 [31]. This calculation allowed us to test for thermally induced effects and impact of larval age in real time and at specific developmental stages.

Statistical analysis

To compensate for the thermal effect on development, we analysed the influence of temperature on gene expression during standardized developmental intervals and during larval development in "real time". Statistical models were used to investigate temperature effects on larval morphology and gene expression throughout early larval development (0 to 18 days post hatch (dph)) and at specific developmental stages (Stages 1-3). Across the temperature treatments, Stage 1 represents the day of hatch, Stage 2 represents the timing of teeth formation, and Stage 3 represents the first-feeding stages [23].

To examine the effect of temperature on gene expression in real time, we used two statistical approaches. In the first approach, we analysed the data using a series of repeated measures mixed-model ANOVAs (PROC MIXED; SAS Institute 2003). Models contained the temperature (16, 18, 20 and 22°C) and age (0 to 18 DPH) or stage (1, 2 and 3) main effects as well as the temperature × age (or stage) interaction term. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate [32]. Temperature and age (or stage) were considered fixed, whereas parental cross was considered random. Tukey's post-hoc analyses were used to compare means between treatments. If a significant temperature × age (or stage) interaction was detected, the model was decomposed into a series of reduced ANOVA models to determine the effect of temperature within each age (or stage) and of age (or stage) for each temperature on gene expression. This was the case for *il1β*, *lysc*, *irf7*, and *mhc2*. Reduced models involved only pre-planned comparisons and did not include repeated use of the same data, so alpha level corrections for *a posteriori* comparisons were not necessary.

In the second approach, we examined variation in gene expression, throughout development at each temperature, by fitting linear, quadratic, or cubic equations (PROC REG; SAS Institute 2003). This allowed us to create predictive models to explore patterns of variation throughout early development at each temperature. Linear, quadratic, or cubic equations were chosen a-priori to fit the data [33]. Final equation selection (linear, quadratic, or cubic) was based on an F-statistic: $d.f._j \times (R^2j - R^2i)/(1 - R^2j)$, where: R^2i = the R^2 for the i-th order, R^2j = the r^2 for the next higher order, $d.f._j$ = the degrees of freedom for the higher-order equation with j degrees of freedom in the numerator and $d.f._j = n - j - 1$ degrees of freedom in the denominator [33]. Graphs and regressions were prepared in SigmaPlot® (Version 13.0).

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Author's contribution

Conceived and designed the larval experiments: SNP, IAEB, JT. Primary funding acquisition: JT, IAEB. Provided eggs for the experiment, JT, IAEB. Performed the larval experiments: SNP, IAEB. Resources for the experimental work: JT, IAEB. Contributed reagents/materials/analysis tools: JJM, IAEB, JT. Performed molecular analysis: JJM, SNP. Analysed the data: JJM, SNP, IAEB. Writing - original draft preparation: JJM and SNP. Writing - Review and editing: IAEB and JT. All authors gave final approval for publication.

Additional information

Competing financial interests: The authors declare that they have no competing or financial interests. **Data availability:** The data for this study will be archived in the repository PANGEA following best practices [60] and will be available upon acceptation of the manuscript.

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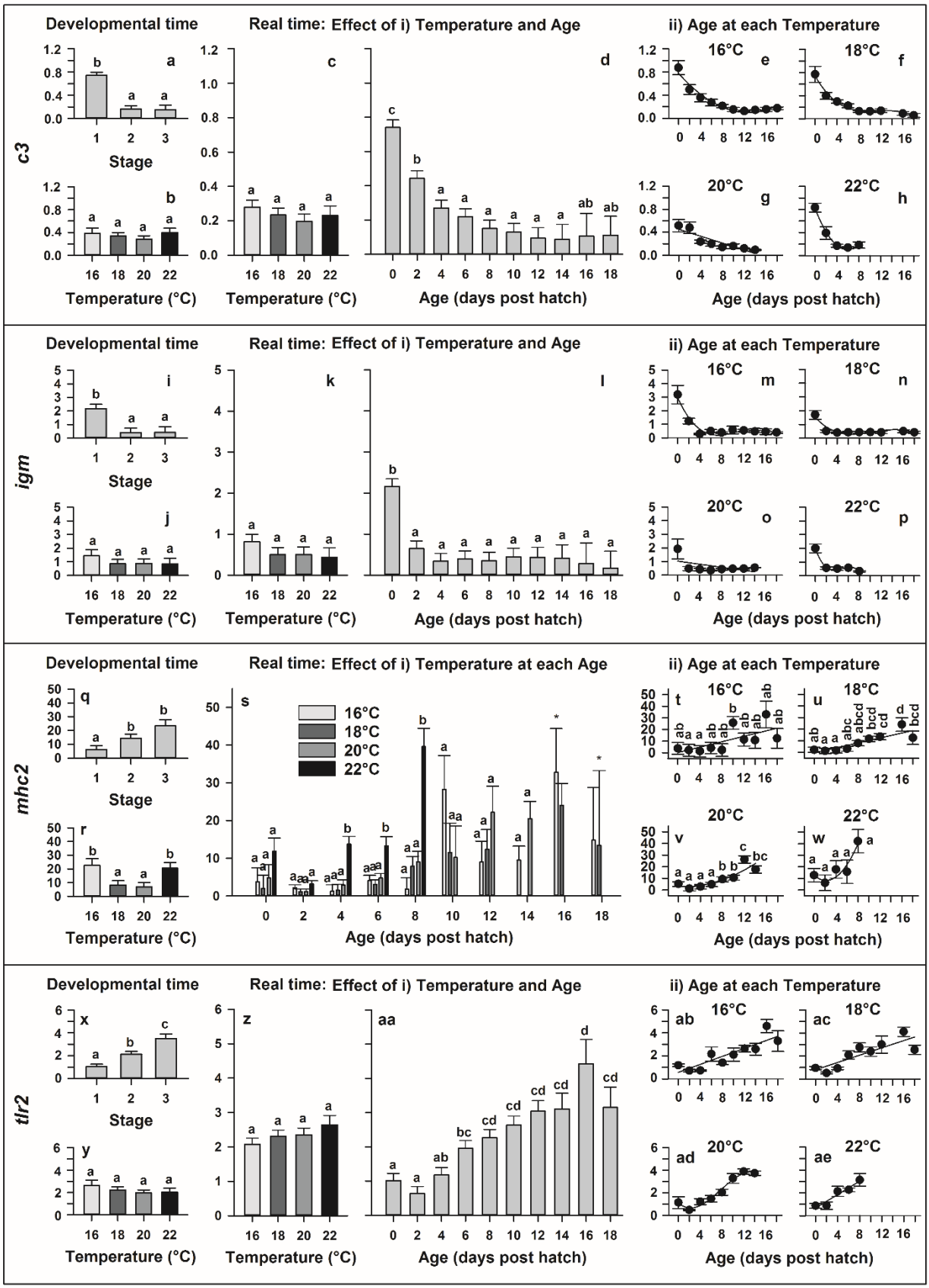
Developmental time
Real time: Effect of i) Temperature and Age
ii) Age at each Temperature
c3
igm
mhc2
tlr2
Stage
Temperature (°C)
Age (days post hatch)
16°C
18°C
20°C
22°C

Fig. 1: Effect of temperature on *c3, igm, mhc2, and tlr2* gene expression of European eel (*Anguilla anguilla*) larvae. The effect of temperature on relative expression of *c3* (a-h), *igm* (i-p), *mhc2* (q-w), and *tlr2* (x-ae) was compared in developmental time (at specific developmental stages (1-3)) or real time (0 to 18 days post hatch). Stage 1 represents the day of hatch, Stage 2 represents the timing of teeth formation, and Stage 3 represents the first-feeding stage. Data points with an asterisk (*) were not included in the statistical model. Values represent means (±SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

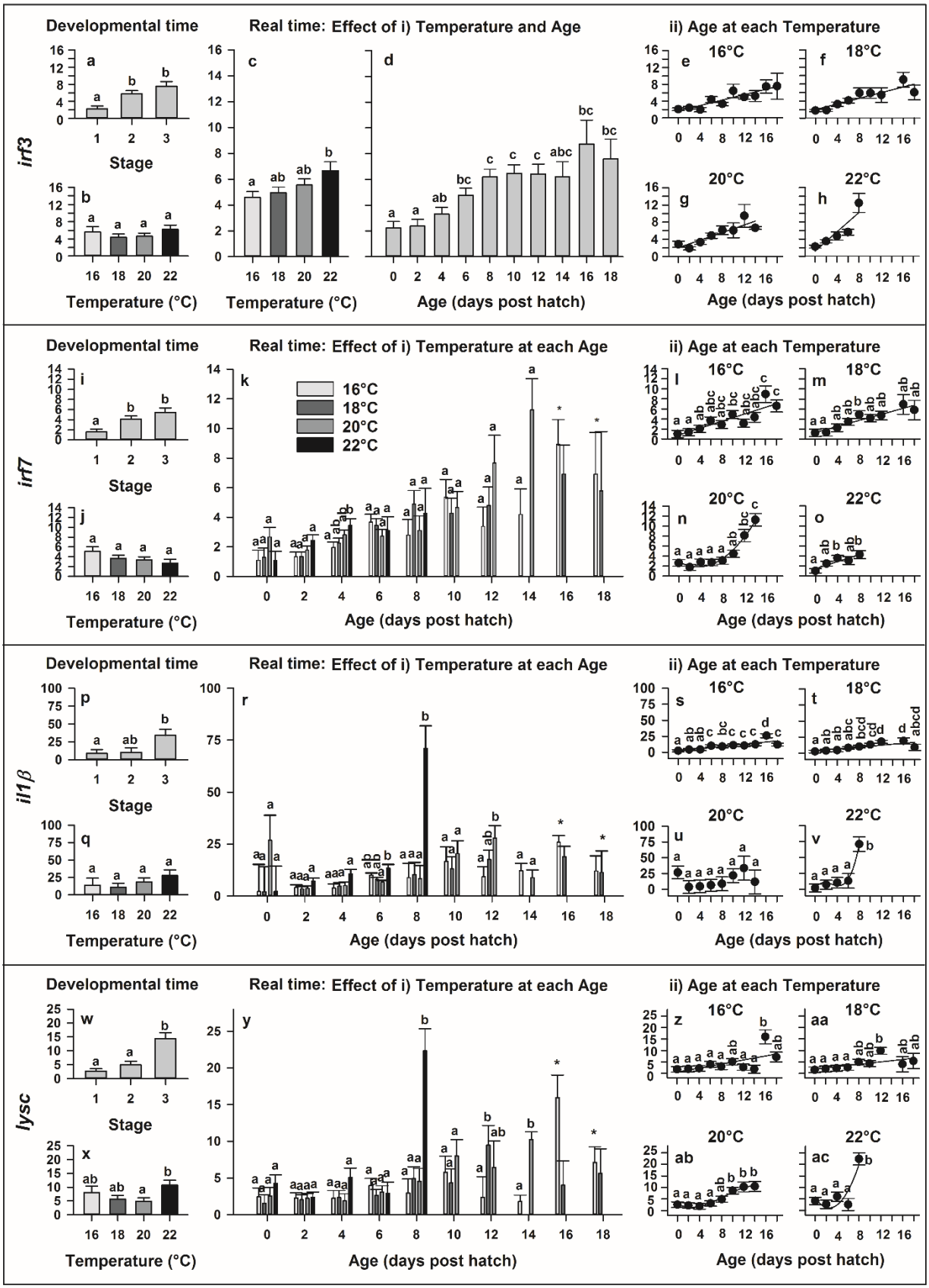
irf3
irf7
il1β
lysc
Developmental time
Real time: Effect of i) Temperature and Age
Real time: Effect of i) Temperature at each Age
ii) Age at each Temperature
Stage
Temperature (°C)
Age (days post hatch)
16°C
18°C
20°C
22°C

Fig. 2: Effect of temperature on *irf3, irf7, il1β, and lysc* gene expression of European eel (*Anguilla anguilla*) larvae. The effect of temperature on relative expression of *irf3* (a-h), *irf7* (i-o), *il1β* (p-v), and *lysc* (w-ac) was compared in developmental time (at specific developmental stages (1-3)) or real time (0 to 18 days post hatch). Stage 1 represents the day of hatch, Stage 2 represents the timing of teeth formation, and Stage 3 represents the first-feeding stage. Data points with an asterisk (*) were not included in the statistical model. Values represent means (±SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

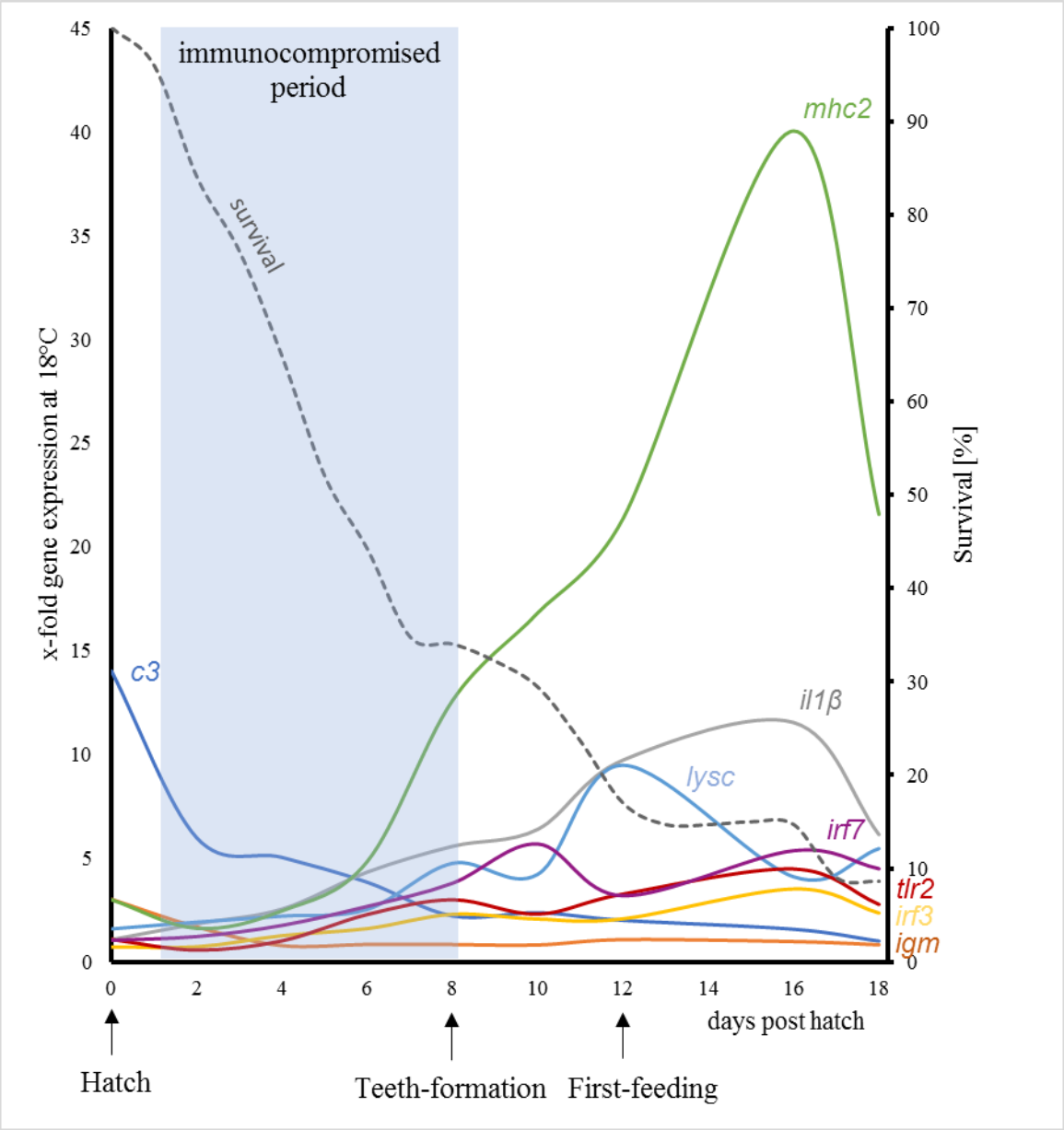

Fig. 3: Conceptual overview - Immunocompromised window during larval development of European eel at 18°C linked to survival. Expression ($2^{-\Delta\Delta ct}$) was calculated in relation to the lowest mRNA level. For variation see Fig. 1-2. Survival (grey dashed line) is displayed on the right axis. For survival: Larvae from 12 parental combinations were stocked at ~150 larvae/L in a recirculation aquaculture system equipped with ~45 L aquaria (50 × 30 × 35 cm), UV sterilizer, protein skimmer, and biofilter. Larvae were reared at 18-20°C in 36 psu filtered (1-10 µm) North Sea seawater supplemented with artificial salt. The number of surviving larvae was estimated per aquarium by subjective estimate and recorded.

Study 4:

Salinity reduction benefits European eel larvae: Insights at morphological and molecular level

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RESEARCH ARTICLE

Salinity reduction benefits European eel larvae: Insights at the morphological and molecular level

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Abstract

European eel (*Anguilla anguilla*) is a euryhaline species, that has adapted to cope with both, hyper- and hypo-osmotic environments. This study investigates the effect of salinity, from a morphological and molecular point of view on European eel larvae reared from 0 to 12 days post hatch (dph). Offspring reared in 36 practical salinity units (psu; control), were compared with larvae reared in six scenarios, where salinity was decreased on 0 or 3 dph and in rates of 1, 2 or 4 psu/day, towards iso-osmotic conditions. Results showed that several genes relating to osmoregulation (*nkcc2α*, *nkcc2β*, *aqp1dup*, *aqpe*), stress response (*hsp70*, *hsp90*), and thyroid metabolism (*thrαA*, *thrαB*, *thrβB*, *dio1*, *dio2*, *dio3*) were differentially expressed throughout larval development, while *nkcc1α*, *nkcc2β*, *aqp3*, *aqp1dup*, *aqpe*, *hsp90*, *thrαA* and *dio3* showed lower expression in response to the salinity reduction. Moreover, larvae were able to keep energy metabolism related gene expression (*atp6*, *cox1*) at stable levels, irrespective of the salinity reduction. As such, when reducing salinity, an energy surplus associated to reduced osmoregulation demands and stress (lower *nkcc*, *aqp* and *hsp* expression), likely facilitated the observed increased survival, improved biometry and enhanced growth efficiency. Additionally, the salinity reduction decreased the amount of severe deformities such as spinal curvature and emaciation but also induced an edematous state of the larval heart, resulting in the most balanced mortality/deformity ratio when salinity was decreased on 3 dph and at 2 psu/day. However, the persistency of the pericardial edema and if or how it represents an obstacle in further larval development needs to be further clarified. In conclusion, this study clearly showed that salinity reduction regimes towards iso-osmotic conditions facilitated the European eel pre-leptocephalus development and revealed the existence of highly sensitive and regulated osmoregulation processes at such early life stage of this species.

resources. The need to harmonize and standardize evolving methodologies, and improve transfer from academia to industry; AQUAGAMETE). Financial support for Ian A.E. Butts was partially provided by the Alabama Agricultural Experiment Station and the Hatch program of the National Institute of Food and Agriculture, US Department of Agriculture. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Eels (*Anguilla spp*.) are euryhaline species that have adapted to cope with both, hyper- and hypo-osmotic environments, likely due to regular salinity changes in their habitats (i.e. estuaries) and/or migrations between freshwater and marine environments to complete their life cycle [1]. Adult eels undertake a downstream (catadromous) reproductive migration towards oceanic spawning areas, while eel offspring undertake an upstream migration towards estuarine or freshwater environments [1]. Unfortunately though, the natural populations and their reproductive potential have declined to a historical minimum, mainly due to climatic and anthropogenic pressures during the different phases of the eel life cycle [2]. The most exploited and negatively impacted populations today are the endangered American (*A. rostrata*) eel and Japanese (*A. japonica*) eel [3–4] as well as the critically endangered European (*A. anguilla*) eel [5].

In this context, actions have been taken to circumvent the eel decline, by minimizing fishery and pollution pressure through restocking and habitat restoration action plans [2]. Additionally, research is being conducted to advance knowledge on assisted reproduction and subsequent early life history (ELH) rearing conditions, towards a sustainable aquaculture. After extensive efforts, breeding protocols using assisted reproductive technologies were developed for the Japanese eel, leading to the first production of eel leptocephali larvae in captivity [6]. The Japanese eel achievements formed the basis for eel research, which led to the development of artificial reproductive protocols of the American [7] and European eel [8–10]. Subsequent work sought to identify optimal environmental rearing conditions throughout the ELH of eels, such as temperature [11–13], light [14], and salinity [15–16].

Regarding salinity, most fish species are hyper-osmotic in freshwater, where plasma osmolality is higher than the environment and hypo-osmotic in seawater, where plasma osmolality is lower than the environment [17]. Thus, in freshwater fish need to actively take up ions to counteract the diffusive ion loss and osmotic water gain, while in seawater they need to maintain osmotic balance through a desalting process to counteract osmotic water loss [18]. Eel offspring naturally occur in a hypo-osmotic environment in the ocean [19]. Interestingly though, it was shown that reducing salinity during ELH rearing, results in better growth and survival of Japanese eel larvae [15]. In some fish species, osmoregulatory organs such as gills or kidneys are not fully or not at all developed during ELH, thus embryos and early larvae control their ion balance *via* chloride cells, commonly located in the yolk sac area and the epithelia covering the larval body surface [20–21]. Those extra-branchial chloride cells (ionocytes) were also found to be located in the epithelium of the Japanese eel larval body surface and particularly abundant in the abdominal region, while forming multicellular complexes and influencing ionoregulation during ELH [22]. Furthermore, it was discovered that Japanese eel larvae can drink as early as the day of hatch, revealing that the role of gastro-intestinal osmoregulation starts earlier than previously anticipated [16, 23, 24]. The role and timing of the intestine and rectum in controlling ion balance was further confirmed by expression of osmoregulatory related genes such as Na^+ K^+ Cl^- (*nkcc*) and Na^+ Cl^- (*ncc*) cotransporters in Japanese eel larvae [16].

Moreover, a recent study, using a transcriptomic (microarray) approach allowed the identification of a large number of genes involved with or affected by osmoregulatory changes during salinity adaptation in the European eel [25]. Furthermore, the European eel genome was recently sequenced and assembled [26], offering new perspectives for eel research in order to gain further knowledge regarding the molecular biology of this species. Thus, it is increasingly possible to follow targeted expression of genes involved in specific molecular mechanisms such as sodium potassium chloride ion cotransporters, which mediate the electroneutral

cotransport of Na^+, K^+ and Cl^- and are known to be involved in ion absorption and/or secretion as well as in cell volume homeostasis [27]. An additional mechanism of interest involves aquaporins, which are membrane proteins, forming pores to selectively facilitate rapid transport and exchange of water molecules in addition to diffusion through the plasma membrane [28]. Also an important mechanism controlling cellular homeostasis, is the molecular response to extrinsic (environmental) stressors. Here, heat shock proteins which are produced in all cellular organisms, regulate normal protein synthesis, and play an important role in an organism's health as they are expressed in response to stress in order to counteract physiological injury and reduce trauma [29]. A different molecular mechanism of interest is the thyroid endocrine system, as it has been shown to be involved in various physiological processes and contributes to homeostasis regulation [30]. Furthermore, the oxidative phosphorylation (OXPHOS) pathway represents a mechanism of importance, where genes associated to energy metabolism are today well known in teleost fishes [31].

In this context, this study investigates how salinity affects European eel larval biometry (such as morphology and growth), deformities and survival as well as expression profiles of genes related to processes involved in or affected by osmoregulation, such as ion transport [$Na^+K^+2Cl^-$ cotransporters (*nkcc*), aquaporins (*aqp*)], energy metabolism [mitochondrial ATP Synthase F0 subunit 6 (*atp6*), cytochrome C oxidase 1 (*cox1*)], thyroid metabolism [thyroid hormone receptors (*thr*), deiodinases (*dio*)] and stress response [heat shock proteins (*hsp*)]. We hypothesized that lower salinity towards a more iso-osmotic environment, already at a very early stage, would reduce stress and conserve energy due to lower cost for osmoregulation, resulting in higher survival and growth during the ELH stages but also facilitating a greater larval developmental potential. Thus, the main objective of this study was to determine the optimal condition for rearing European eel pre-leptocephalus larvae by decreasing salinity on 0 or 3 days post hatch (dph) and at rates of 1, 2 or 4 psu/day.

Material and methods

Ethics statement

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental 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. Endogenously feeding larvae of European eel were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222). All efforts were made to minimize animal handling and stress.

Broodstock management

Female broodstock were wild-caught from lake Vandet (Denmark) or Lough Neagh (Ireland), while all males originated from a commercial eel farm (Stensgård Eel Farm A/S, Denmark). After collection, broodstock were transferred to an experimental facility of the Technical University of Denmark, where they were maintained in ~1250 L polyethylene tanks equipped with a closed recirculation system, under a continuous flow rate of ~10–15 L min^{-1}, low intensity light (~20 lux) and 12 h day/12 h night photoperiod. Acclimatization took place over two weeks, in order 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, experimental design and conditions

To induce vitellogenesis females received weekly injections of salmon pituitary extract at 18.75 mg kg^{-1} body weight (Argent Chemical Laboratories, Washington, USA) [10, 32]. To stimulate follicular maturation and induce ovulation, females received an additional injection of 17α,20ß-dihydroxy-4-pregnen-3-one (Sigma-Aldrich, St. Louis, MO, USA) at 2.0 mg kg^{-1} body weight [33]. Then, within 12–14 h, eggs were stripped from females. Males received weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich Chemie, Steinheim, Germany) at 150 IU/fish. Prior to fertilization, they were given another injection and milt was collected ~12 h after administration of hormone. Milt samples were pipetted into an immobilizing medium [34] and used for fertilization within 4 h of collection [35].

The experiment was repeated 3 times, each time using a different experimental cross. Eggs from each female were "crossed" with a sperm pool of several males to experimentally create the 3 experimental crosses. Eggs from each female were stripped into dry plastic containers and gametes were swirled together. Artificial seawater (20˚C and 36 psu) prepared by using reverse osmosis filtration (Vertex Puratek 100 gpd RO/DI, Vertex Technologies Inc., CA, USA) and salted with Red Sea Salt (Red Sea, Red Sea International, Eilat, Israel) was added for a gamete contact time of 5 min [35, 36]. Eggs/embryos were then incubated in 15 L of the above described artificial seawater (18˚C and 36 psu) [13] and supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) until hatch [37].

At hatch, larvae were randomly distributed (~1000 individuals per replicate) into 1 L glass beakers and reared throughout the endogenous feeding stage, from 0 to 12 dph, with no aeration [38]. Each beaker was filled with 800 mL of artificial seawater (18˚C and 36 psu) and supplemented with antibiotics as previously described [13, 37]. Using a factorial approach, salinity treatments were step-wise adjusted beginning on 0 or 3 dph and at rates of 1, 2, or 4 psu/day, in order to reach a more iso-osmotic condition compared to full strength seawater (control), which was kept at 36 psu. This resulted in overall seven salinity treatments (con, 01, 02, 04, 31, 32 and 34) described in Table 1. All 3 experimental crosses where represented in 3 replicates for all 7 treatments, resulting in 63 experimental beakers. Each day, 400 mL were removed and replaced with pre-adjusted artificial seawater, in order to reach the desired salinity of each treatment, which was measured using a digital portable conductivity meter (WTW ProfiLine Cond 3110). All treatments underwent the same handling procedures. Rearing of embryos and larvae took place in darkness, while handling and sampling under low intensity (< 2.2 μmol m^{-2} s^{-1}) light conditions [14].

Table 1. European eel (*Anguilla anguilla*) larvae reared in seven different salinity treatments; at 36 psu (con) and in six further scenarios, where salinity was reduced on 0 or 3 days post hatch and at rates of 1, 2 or 4 psu/day (01, 02, 04, 31, 32 and 34) towards iso-osmotic conditions.

			Age in days post hatch (dph)												
Reduction		ID	0	1	2	3	4	5	6	7	8	9	10	11	12
dph	psu day^{-1}		Salinity (psu)												
Control		con	36												
0	1	01	35	34	33	32	31	30	29	28	27	26	25	24	23
	2	02	34	32	30	28	26	24	22	20	18	16			
	4	04	32	28	24	20	16								
3	1	31	36			35	34	33	32	31	30	29	28	27	26
	2	32	36			34	32	30	28	26	24	22	20	18	16
	4	34	36			32	28	24	20	16					

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Data collection

Larval development (biometry), mortality, and gene expression of selected genes, corresponding to specific molecular mechanisms were followed from hatch and throughout the endogenous feeding stage (2, 4, 6, 8, 10, and 12 dph). All endogenously feeding larvae of European eel 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 [13]. 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 analyze the larval images (Nikon Corporation, Japan).

Mortality and biometry. Every day, dead larvae were counted and removed from all experimental units. Additionally, all larvae at the end of the experiment as well as of all sampled larvae from each experimental unit were enumerated and recorded. Larval cumulative mortality was calculated as a percentage from hatch until 12 dph.

For analysis of larval biometry, ~10 larvae from each replicate (3×), cross, and treatment were randomly sampled at hatch and every second day until 12 dph. Larvae were digitally imaged (as described above) for later analyses, where total body and oil-drop area were measured for each larva. Larval growth and oil-drop utilization were measured from the change in body and oil-drop area, respectively. Growth efficiency was measured by dividing the increase in body area by the corresponding decrease in oil-drop area. Moreover, larval deformities were classified according to [39] as spinal curvature, emaciation and pericardial edema (Fig 1).

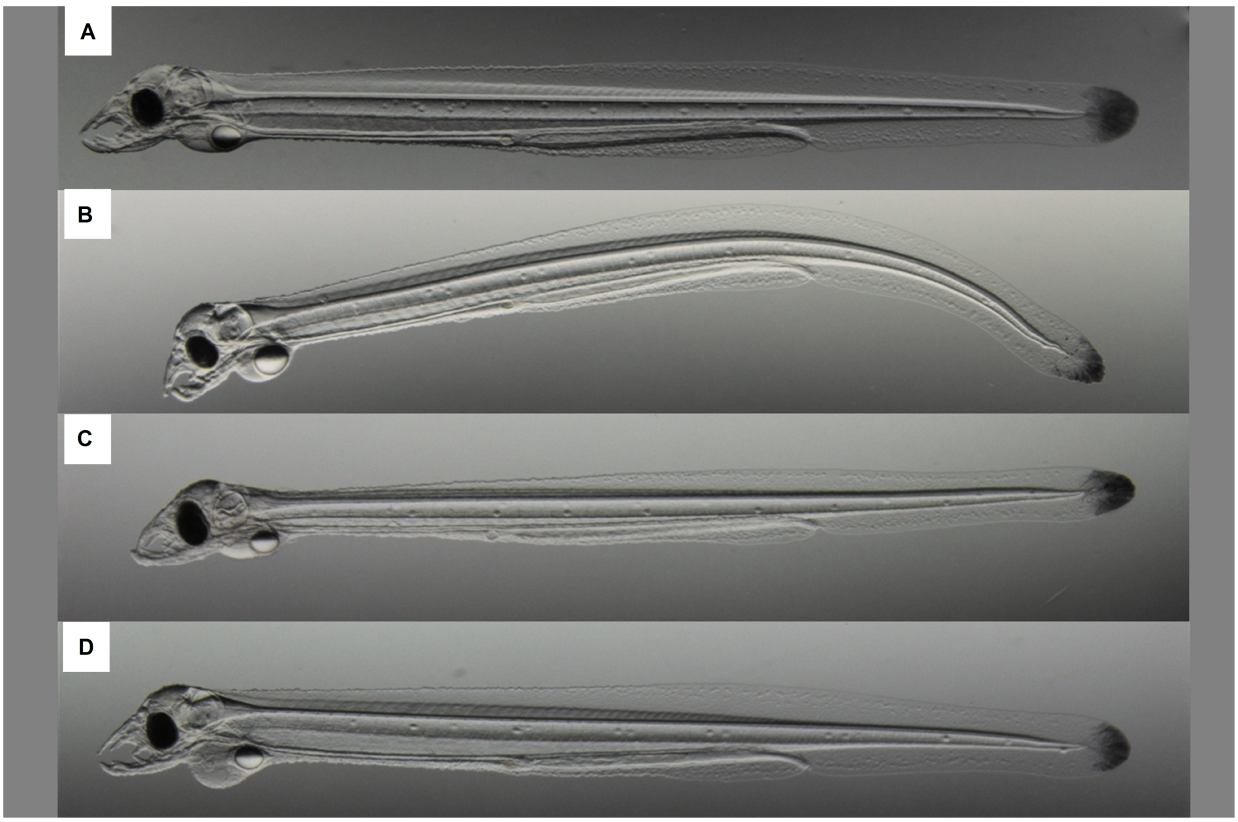

Fig 1. Visualization of European eel (*Anguilla anguilla*) larval deformities. Normal (A), spinal curvature (B), emaciation (C), and pericardial edema (D).

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Gene expression. For molecular analysis, ~30 larvae from each replicate, experimental cross, and treatment were randomly sampled at hatch and every second dph until the first-feeding stage. Those larvae were recorded, euthanized using MS-222, rinsed with deionized water, preserved in RNAlater Stabilization Reagent and kept at -20˚C following the procedure suggested by the supplier (Qiagen, Hilden, Germany). RNA was extracted using the NucleoSpin® RNA Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. RNA concentration (264 ± 230 ng μl^{-1}) and purity (260/280 = 2.13 ± 0.03, 260/230 = 2.23 ± 0.12) were determined by spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany) and normalized to a common concentration of 100 ng μl^{-1} with HPLC water. From the resulting total RNA, 680 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 transcription [PerfeCta® DNase I Kit (Quantabio, Germany)].

The *ef1α* and rps*18* genes were chosen as housekeeping genes, since 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 [40]. The expression levels of 16 target and 2 reference (*ef1α*, rps*18*) genes were determined by quantitative real-time PCR (RT-qPCR), using specific primers (Table 2). Primers were designed using primer 3 software v 0.4.0 (http://frodo.wi.mit.edu/primer3/) based on cDNA sequences available in GenBank Nucleotide, the European eel transcriptome database (EeelBase 2.0, http://compgen.bio.unipd.it/eeelbase/) or the available European eel genome (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA73577). 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 2 randomly selected replicates, from each parental cross, treatment, and larval age (2, 4, 6, 8, 10 and 12 dph) were analysed in two technical replicates of each gene using the qPCR Biomark™ HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips) as previously described [41]. 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 μL cDNA per sample for 10 min at 95˚C and then 14 cycles of 15 s 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 and measured using the ΔΔ Ct method [42]. Coefficient of variation (CV) of technical replicates was calculated and checked to be < 0.04 [40].

Statistical analyses

All data were analyzed using SAS statistical software (version 9.1; SAS Institute Inc., Cary, North Carolina). Residuals were tested for normality using the Shapiro-Wilk test and homogeneity of variances was tested using a plot of residuals versus fit values (PROC GLOT, SAS Institute 2003). Data were $\log_{10}$ or arcsine square-root-transformed when data deviated from normality or homoscedasticity [43]. The effect of salinity on larval deformities, growth rate, oil-drop utilization, and growth efficiency on 12 dph was determined using a series of one-way ANOVA models, where experimental cross was considered a random factor (SAS PROC MIXED; SAS Institute 2003). Tukey's post-hoc analyses were used to compare least-squares means between treatments. Furthermore, statistical models were used to investigate salinity effects on larval body area, mortality and gene expression throughout early larval development (from 2 to 12 dph). Here, we analyzed the data using a series of repeated measures mixed-

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. The table lists accession number and corresponding database of target gene sequences.

Full name	Abbreviation	Function	Database	Accession Number	Primer sequence (5' 3') (F: Forward; R: Reverse)
$Na^+K^+2Cl^-$ Cotransporter 1α	*nkcc1α*	Ion transport	GenBank Nucleotide	AJ486858	F: CCAAGGCTCAGATCTTCCTG
					R: TTTCCGAATGGTAACCGAAG
$Na^+K^+2Cl^-$ Cotransporter 2α	*nkcc2α*	Ion transport	GenBank Nucleotide	AJ564602	F: ACGTGGTTGGGTTTTCAGAG
					R: GTGAGATCCCCAAAAGCAAA
$Na^+K^+2Cl^-$ Cotransporter 2β	*nkcc2β*	Ion transport	GenBank Nucleotide	AJ564603	F: AGCCAAAGTGGTGGATGTTC
					R: TGTCAGCCTCTCCAGTTCCT
Aquaporin 3	*aqp3*	Water transport	GenBank Nucleotide	AJ319533	F: GCTCTCATGGCTTGTTCCTC
					R: AAGGTCACAGTGGGGTTCAG
Aquaporin 1 duplicate	*aqp1dup*	Water transport	GenBank Nucleotide	AJ564421	F: GAATTCCTGGCAACCTTTCA
					R: CAAGATGACCCAGACCCACT
Aquaporin e	*aqpe*	Water transport	GenBank Nucleotide	AJ784153	F: TGGGCAGCTGACAGTAACAG
					R: AATCACCTGGTCCACAAAGC
Heat Shock Protein 70	*hsp70*	Stress response	GenBank WGS	AZBK01685255	F: TCAACCCAGATGAAGCAGTG
					R: GCAGCAGATCCTGAACATTG
Heat Shock Protein 90	*hsp90*	Stress response	GenBank WGS	AZBK01838994	F: ACCATTGCCAAGTCAGGAAC
					R: ACTGCTCATCGTCATTGTGC
ATP Synthase F0 subunit 6	*atp6*	Energy metabolism	GenBank Nucleotide	NC_006531	F: GGCCTGCTCCCATACACATT
					R: GACTGGTGTTCCTTCTGGCA
Cytochrome C Oxidase 1	*cox1*	Energy metabolism	GenBank Nucleotide	NC_006531	F: CTACTCCTCTCCCTGCCAGT
					R: CTTCTGGGTGGCCGAAGAAT
Deiodinase 1	*dio1*	Thyroid metabolism	EeelBase 2.0	Eeel2-c186	F: AGCTTTGCCAGAACGACTGT
					R: TTCCAGAACTCTTCGCACCT
Deiodinase 2	*dio2*	Thyroid metabolism	Eel genome website	g12347	F: GAAGAGGAGGATCGCCTACC
					R: GCACTCTACCTCCGTCCAAA
Deiodinase 3	*dio3*	Thyroid metabolism	EeelBase 2.0	Eeel-c22164	F: TACGGGGCGTATTTTGAGAG
					R: GCTATAACCCTCCGGACCTC
Thyroid Hormone Receptor α A	*thrαA*	Thyroid metabolism	GenBank Nucleotide	KY082904	F: GCAGTTCAACCTGGACGACT
					R: CCTGGCACTTCTCGATCTTC
Thyroid Hormone Receptor α B	*thrαB*	Thyroid metabolism	GenBank Nucleotide	KY082905	F: GAAGCCTTCAGCGAGTTCAC
					R: ACAGCCTTTCAGGAGGATGA
Thyroid Hormone Receptor β B	*thrβB*	Thyroid metabolism	GenBank Nucleotide	KY082907	F: GAAGACTGAGCCCTGAGGTG
					R: AGGTAATGCAGCGGTAATGG
Elongation Factor 1 α	*ef1a*	Housekeeping	GenBank Nucleotide	EU407824	F: CTGAAGCCTGGTATGGTGGT
					R: CATGGTGCATTTCCACAGAC
40S Ribosomal S18	*rps18*	Housekeeping	GenBank TSA	GBXM01005349	F: TGACCGATGATGAGGTTGAG
					R: GTTTGTTGTCCAGACCGTTG

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model ANOVAs (PROC MIXED; SAS Institute 2003). Models contained salinity treatment and age main effects as well as the treatment × age interaction. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate [44]. Salinity treatment and age were considered fixed, whereas parental cross was considered random. Tukey's post-hoc analyses were used to compare least-squares means between treatments. If a significant salinity × age interaction was detected, the model was decomposed into a series of reduced ANOVA models to determine the effect of salinity for each age. This was the case only for *thrαA*.

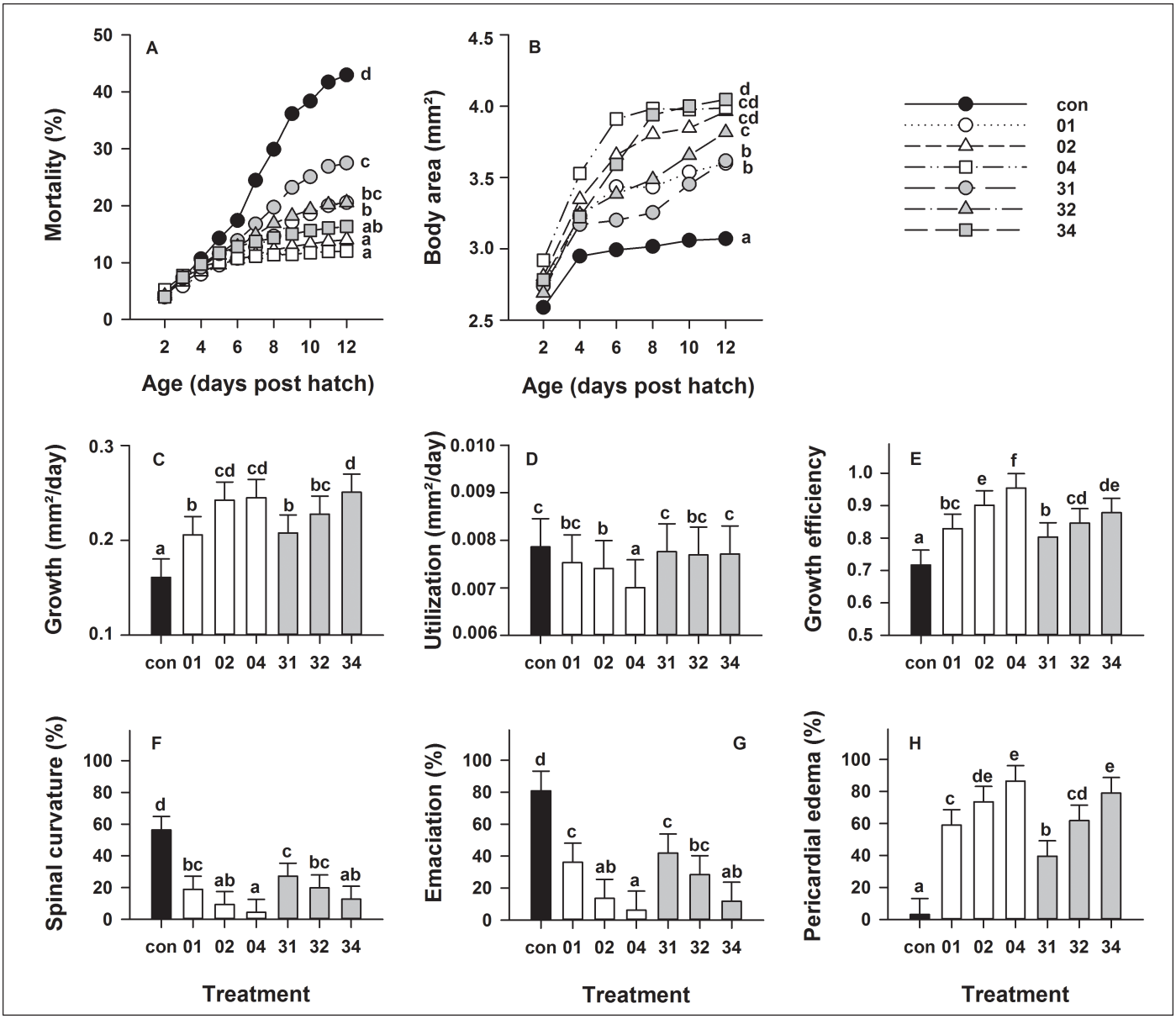

Fig 2. Effect of salinity on European eel (*Anguilla anguilla*) larval mortality and biometry. Mortality (A), body area (B), growth (C), oil drop utilization (D) and growth efficiency (E) from 2 to 12 days post hatch (dph) as well as occurrence of larval deformities such as spinal curvature (F), emaciation (G) and pericardial edema (H) on 12 dph. Values represent means (± SEM) among three crosses at each age and treatment. Different lower case letters represent significant statistical differences ($p < 0.05$).

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Results

Mortality and biometry

European eel larval mortality (± SEM) until 12 dph, was highest (43 ± 10%) when larvae were reared at 36 psu (control) and significantly ($p < 0.0001$) lower in all salinity reduced treatments (Fig 2A). The statistically lowest larval mortality was observed when salinity was initially reduced on 0 dph and at 2 or 4 psu/day (treatments 02 and 04, respectively) as well as on 3 dph and at 4 psu/day (treatment 34).

Larval body area (±SEM), which increased until 12 dph, was found to be lowest (3.1 ± 0.2 mm^2) when larvae were reared at 36 psu (control) and significantly ($p < 0.0001$) higher in all salinity reduced treatments (Fig 2B). Larvae developed the highest body area (3.8–4.0 ± 0.2 mm^2) when salinity was initially reduced on 0 dph and at 2 or 4 psu/day (treatments 02 and 04, respectively) as well as on 3 dph and at 4 psu/day (treatment 34).

Larval growth (± SEM) throughout the endogenous feeding window was significantly ($p < 0.0001$) lower (0.16 ± 0.02 mm^2 per day) when larvae were reared at 36 psu (control) compared to all other treatments (Fig 2C). Highest growth (> 0.24 ± 0.02 mm^2/day) occurred when salinity was reduced on 0 dph and at 2 or 4 psu/day (treatments 02 and 04, respectively), as well as on 3 dph and at 4 psu/day (treatment 34). Oil drop utilization (± SEM) was significantly ($p < 0.0001$) lower (0.007 ± 0.0006 mm^2/day) when salinity was reduced on 0 dph and at 4 psu/day (treatment 04) compared to all other treatments (Fig 2D). All salinity reduction treatments resulted in a growth efficiency (± SEM) that was significantly ($p < 0.0001$) higher compared to larvae reared at 36 psu (control; 0.72 ± 0.04), while highest growth efficiency (0.95 ± 0.04) occurred when salinity was reduced on 0 dph and at 4 psu/day (treatment 04; Fig 2E).

On 12 dph, the occurrence of larvae with spinal curvature (± SEM) were significantly ($p < 0.0001$) higher (56 ± 8%) when larvae were reared at 36 psu (control), compared to all salinity reduction treatments (Fig 2F), while lowest (4 ± 8%) occurrence of larvae with spinal curvature were detected when salinity was reduced on 0 dph and at 4 psu/day (treatment 04). Similarly, the occurrence of larvae with emaciation (± SEM) were significantly ($p < 0.0001$) higher (81 ± 12%) when larvae were reared at 36 psu (control), compared to all salinity reduction treatments (Fig 2G), while lowest (6 ± 8%) occurrence of larvae with emaciation were detected when salinity was reduced on 0 dph and at 4 psu/day (treatment 04). On the contrary, when salinity was reduced on 0 dph and at 2 or 4 psu/day (treatments 02 and 04, respectively), as well as on 3 dph and at 4 psu/day (treatment 34), we observed larvae with a significantly ($p < 0.0001$) higher (> 73 ± 10%) number of pericardial edema compared to larvae reared at 36 psu (control; Fig 2H).

Gene expression

Osmoregulation. Na^+ K^+ $2Cl^-$ cotransporter regulation were affected by both larval age and salinity. Relative expression levels of the genes encoding for *nkcc1α* were stable (Fig 3A), while *nkcc2α* and *nkcc2β* increased significantly ($p < 0.0001$) throughout larval development and peaked at 12 dph (Fig 3C and 3E). Expression of *nkcc1α* was significantly ($p < 0.01$) reduced when salinity was decreased on 0 dph and at 1 or 4 psu/day (treatments 01 and 04, respectively) as well as on 3 dph and at 4 psu/day (treatment 34; Fig 3B) compared to the 36 psu control. Similarly, expression of *nkcc2β* was significantly ($p < 0.01$) higher in the control group than when salinity was decreased on 0 dph and 4 psu/day (treatment 04; Fig 3F), while no statistically significant effect of salinity was observed on expression levels of *nkcc2α* (Fig 3D).

Moreover, the relative expression of aquaporin 3 (*aqp3*) was stable (Fig 3G), *aqp1dup* significantly ($p < 0.0001$) decreased (Fig 3I), while *aqpe* significantly ($p < 0.0001$) increased throughout larval development (Fig 3K). Expression of *aqp3* was significantly ($p = 0.005$) reduced when salinity was decreased on 0 dph and at 4 psu/day (treatment 04; Fig 3H) compared to no reduction (control) or the slowest reduction in treatment 31 (reduction on 3 dph and at 1 psu/day). Expression of *aqp1dup* was also significantly ($p < 0.002$) higher when larvae were reared at 36 psu (control) compared to when salinity was decreased on 0 dph and at 2 or 4 psu/day (treatments 02 and 04, respectively; Fig 3J). Similarly, the salinity reduction on 0

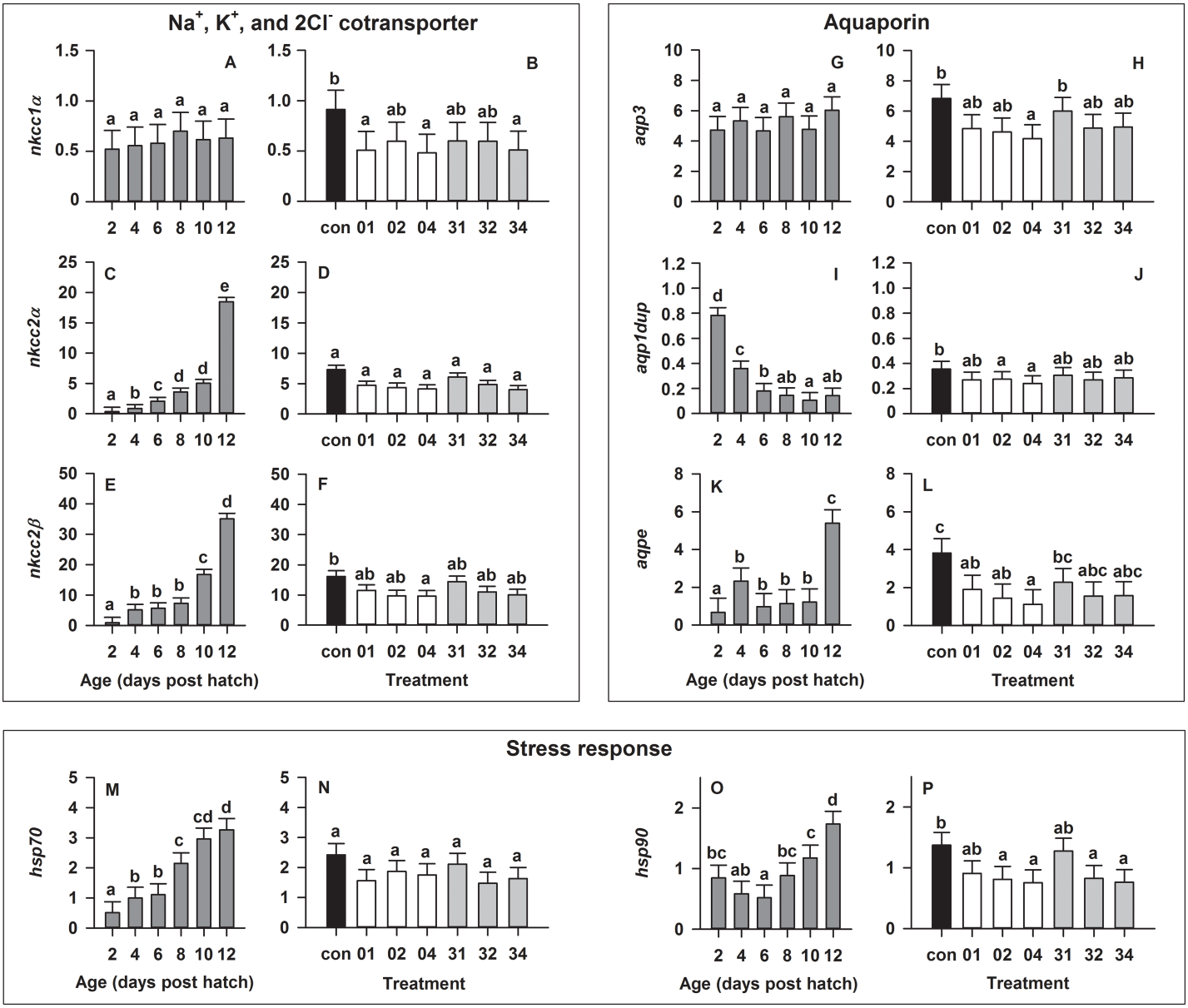

Fig 3. Effect of age and salinity treatment on European eel (*Anguilla anguilla*) larval relative expression of selected genes. No significant salinity × age interaction was detected for any gene. As such, over the entire experimental period, the main effects of age and salinity are displayed for genes encoding $Na^+K^+2Cl^-$ cotransporters (A-F), aquaporins (G-L), and heat shock proteins (M-P). Values represent means (± SEM) among three crosses at each age and treatment. Different lower case letters represent significant statistical differences ($p < 0.05$).

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dph, irrespective of the reduction rate (treatments 01, 02 and 04; Fig 3L), caused a significant ($p < 0.001$) reduction in mRNA levels of the third aquaporin tested (*aqpe*).

Stress response. The relative expression of heat shock protein 70 *(hsp70)* significantly ($p < 0.0001$) increased throughout development, while *hsp90* experienced a significant ($p < 0.0001$) decrease from 2 to 6 dph, but increased again beyond that; both reaching a peak on 12 dph (Fig 3M and 3O). The rate of reduction was the driver setting the expression pattern for *hsp90*, since reduction rates of 2 and 4 psu/day let to lowered mRNA levels independent of the onset of the treatment ($p < 0.001$, Fig 3P). The expression response of *hsp70*

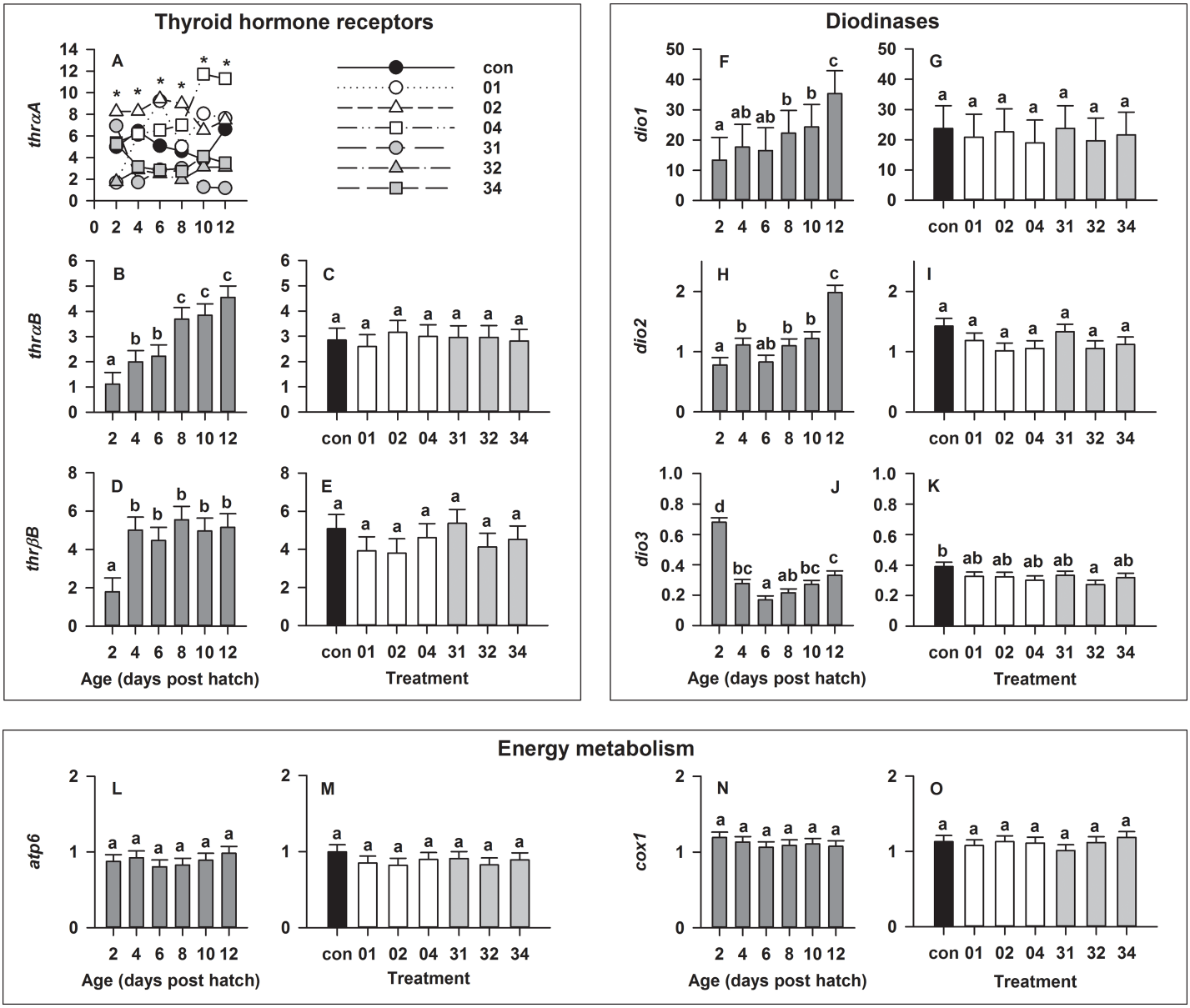

Fig 4. Effect of age and salinity treatment on European eel (*Anguilla anguilla*) larval relative expression of selected genes. For thyroid hormone receptor αA (*thrαA*), a significant salinity × age interaction was detected, thus the model was decomposed into a series of reduced ANOVA models to determine the effect of salinity for each age (A). No significant salinity × age interactions were detected for all other genes. As such, over the entire experimental period, the main effects of age and salinity are displayed for genes relating to thyroid hormone receptors (B-E), deiodinases (F-K), and energy metabolism (L-O). Values represent means (± SEM) among three crosses at each age and treatment. Different lower case letters and asterisks represent significant statistical differences ($p < 0.05$).

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showed a similar pattern to *hsp90*; however it was not statistically different among the treatments (Fig 3N).

Thyroid metabolism. A significant ($p < 0.001$) age × salinity interaction was found for the relative expression of the thyroid hormone receptor *thrαA* (Fig 4A), while *thrαB* and *thrβB* were not significantly affected by salinity (Fig 4C and 4E). For the latter two genes, age was the main factor influencing gene expression. Expression levels of *thrαB* increased throughout ontogeny (Fig 4B), while expression of *thrβB* increased from 2 to 4 dph and remained at steady levels beyond that (Fig 4D; both $p < 0.0001$). Moreover, larval age and salinity influenced

genes involved in deiodination (*dio1-3*). Here, expression of *dio1* and *dio2* increased throughout ontogeny (Fig 4F and 4H), while expression of *dio3* decreased from 2 to 6 dph and increased again beyond that until 12 dph (Fig 4J; all $p < 0.0001$). However, *dio3* was the only gene in this functional group that was influenced by salinity, as its expression was reduced when salinity was decreased on 3 dph and at 2 psu/day ($p < 0.02$; Fig 4K).

Energy metabolism. Energy metabolism related genes (*atp6*, *cox1*) were found to be steadily expressed within the endogenous feeding period (2 to 12 dph) and at all salinities, with no significant differences occurring among treatments (Fig 4L–4O).

Discussion

European eel is critically endangered with diminishing glass eel recruitment [2]. Thus, there is an urgent need to artificially produce offspring and improve survival during ELH of this species. Latest efforts have focused on extrinsic (environmental) factors such as light [14] and temperature [13, 45], while only eggs and embryos have been assessed regarding salinity effects [36]. In the closely related Japanese eel, a major improvement in larval captive rearing has been attributed to reduced salinity regimes [15, 46]. Therefore, this study investigated the effect of salinity on artificially produced European eel larvae (from a morphological and molecular point of view), to elucidate functionality and timing of osmoregulation related processes affecting ELH, in order to ultimately determine optimal conditions for rearing European eel pre-leptocephalus larvae in aquaculture. To accomplish this, we reared offspring throughout the endogenous feeding window (0–12 dph) and compared larvae reared at 36 psu (control), which is close to the salinity occurring in the Sargasso Sea [19], with larvae reared under reduced salinity conditions. Here, salinity was decreased on 0 or 3 dph and at rates of 1, 2 or 4 psu/day, resulting in six different salinity treatments (01, 02, 04, 31, 32 and 34).

Mortality and biometry

Generally, we observed that reducing salinity, especially on 0 dph at 4 psu (fastest reduction) resulted in reduced mortality, increased body area, higher growth, reduced oil drop utilization and improved growth efficiency of the European eel larvae. Similar observations have been reported for Japanese eel, where better larval growth and survival were reported when reared in 50% reduced salinity [15]. Thereafter, it was shown that reducing seawater salinity (with an osmolality of ~1050 mOsm kg^{-1} H_2O) to ~50%, facilitated an iso-osmotic environment for eel larvae with a tissue osmolality of 360 to 540 mOsm kg^{-1} H_2O [23]. Concurrently, it has been argued that reducing salinity probably increased energy availability due to lower osmoregulatory expenses, enabling the survival of weaker larvae, which would not survive in a high salinity environment [46]. Additionally, [47] reported increased spinal deformities at a high salinity level (42 psu). In our study, we observed the highest incidence of spinal curvature (> 50%) and emaciation (> 80%) when larvae were reared at 36 psu (control) compared to < 5% and < 10% respectively, when salinity was reduced on 0 dph and at 4 psu/day (treatment 04). However, it has also been reported that rearing eel larvae at reduced salinity (< 33 psu), causes other deformities, such as pericardial edema and abnormal lower jaw [47]. The results of our study are in agreement with those findings, as we also observed an increased number of larvae with pericardial edema in the salinity reduced treatments. Thus, we demonstrate that reducing salinity was a tradeoff process, which improved survival and growth but also induced an edematous state of the larval heart. Hence, we consider that reducing salinity on 3 dph and at 2 psu/day towards iso-osmotic conditions, results in a more balanced mortality/deformity ratio. However, the persistency of the edematous state of the larval heart and if or how it represents an obstacle in further larval development needs to be clarified *via* future investigations.

Thyroid metabolism

In order to evaluate the underlying molecular backgrounds relating to the observed biometric changes, we targeted genes involved in the thyroid metabolism, which is directly linked to growth and development of fish [48]. The importance of the thyroid endocrine system, especially during metamorphosis, has been documented in the Japanese conger eel, *Conger myriaster* [49], while thyroid hormone treatment is applied to coordinate a synchronized metamorphosis in Japanese eels [50]. Moreover, it was shown that the thyroid hormone pathway is involved in the early life development of Japanese [51] as well as European eels and that genes encoding thyroid hormone receptors and deiodinases show sensitivity to extrinsic (environmental) stimuli, such as temperature [45]. In the current study we observed that an early and fast reduction of salinity from 0 dph onwards increased *thrαA* levels, which is an underlying molecular response that can potentially be correlated to growth efficiency, which was also highest in this treatment. Moreover, the expression of *dio3* was also influenced by the salinity change, which would be in accordance with previous observations, where the outer ring deiodination showed sensitivity to salinity in rainbow trout, *Oncorhynchus mykiss* [52]. Furthermore, all genes relating to thyroid metabolism, investigated in this study, were affected by larval age and showed differentially expressed patterns throughout ontogeny, which are in accordance with previous reported results in European eel larvae [45] and thus further support the involvement of the thyroid endocrine system during ELH of this species.

Osmoregulation

To further support our findings, we followed the relative expression of genes involved in ion transport. Here, we targeted the NKCC subfamily of the chloride cation cotransporter gene family, which mediates the electroneutral cotransport of Na^+, K^+ and Cl^- ions and is known to be involved in ion absorption and/or secretion as well as in cell volume homeostasis [27]. A secretory (*nkcc1*) and an absorptive (*nkcc2*) subtype (with isoforms for each) have been previously identified in adult European eel [53]. Of these, the *nkcc1α* isoform showed a wide range of tissue distribution, whereas *nkcc1β* was predominantly expressed in the brain [54]. Moreover, the *nkcc2α* isoform was rather restricted to renal tissues, whereas the *nkcc2β* isoform predominated in the intestine and urinary bladder [55]. In our study, we observed that *nkcc1α* and *nkcc2β* expression decreased with salinity reduction, which indicates a downregulation of the active Na^+, K^+, and Cl^- transport. As this mechanism requires energy, a lowered transcellular ion transport can probably lead to reduced cellular energy consumption. Furthermore, the ionoregulatory ability of the larvae seemed to increase throughout ontogeny (i.e. increased expression of *nkcc2α* and *nkcc2β*), which is probably coupled to the increasing functionality of the associated tissue (kidney and gut respectively) during organogenesis.

Ionoregulation is tightly coupled to water flow across membranes, where aquaporins form pores to selectively facilitate rapid transport and exchange of water molecules [28]. In euroha-line fish, such as the sea-bass (*Dicentrarchus labrax*), aquaporins facilitate the water uptake in the intestinal tract and the reabsorption of water in the kidney to counteract dehydration in response to high salinity [56]. Similarly, in relation to seawater acclimation, branchial *aqp3* was downregulated, but intestinal *aqp3* was unchanged [57], while renal *aqp1*, *aqp1dup* and *aqpe* were downregulated [58], but intestinal *aqp1* and *aqpe* were upregulated [59] in European eel. Probably the high osmotic water loading through the gills and the associated excretion of large volumes of dilute urine (needed in freshwater) are no longer necessary in a saline environment (gill and renal expression downregulation), while on the contrary, the increased ingestion of seawater and the accompanied transcellular movement of large quantities of salts in the gastro-intestinal track, require concomitant changes in transcellular water transport

(intestinal expression upregulation) [60]. In our study we observed a down-regulation of *aqp1dup* and the two aquaglyceroporins (*aqp3*, *aqpe*) when salinity was reduced early and fast (from 0 dph and at 4 psu/day), which is an indication that larvae acclimated their hydromineral regulation in response to lower salinity by reducing water retention. However, as gills are not present and the kidney as well as the intestine are undeveloped and only gain functionality during this developmental period, the tissue specific functionality of this molecular mechanism in relation to salinity change and the potential sensitivity shift among developmental stages remain to be clarified.

Stress response

In order to analyze the cellular stress response in European eel larvae we targeted *hsp70* and *hsp90*, which are expressed (in response to stress) to counteract physiological injury and reduce trauma [13, 29]. The *hsp* function, is commonly associated but not restricted to heat stress and has been recognized to have a more universal role in response to a number of stressors [29]. As such, *hsps* have been shown to be sensitive to changes in salinity, where lowest levels of *hsps* were found in Black sea bream (*Mylio macrocephalus*) reared in an iso-osmotic salinity and highest levels when reared bellow (hyper-osmotic) or above (hypo-osmotic) the osmotic homeostasis [61]. A similar response was observed in our current study, where larval *hsp90* levels decreased in the treatments were salinity was reduced in 2 or 4 psu/day compared to larvae reared in full strength seawater (36 psu). This suggests that eel larvae reared in iso-osmotic conditions are less stressed probably due to lower energetic costs in maintaining cellular homeostasis.

Energy metabolism

Thereafter, we evaluated energy levels in European eel larvae in response to environmental salinity changes by targeting genes involved in the OXPHOS pathway and associated to energy metabolism in teleost fishes [31]. Here, the expression levels of ATP-synthase and cytochrome-c-oxidase were stable throughout the entire developmental period investigated and independent of salinity levels, suggesting that energy production was stable across all salinity treatments. As such, in the light of decreased osmotic demands and stress (i.e. lower *hsp*, *nkcc*, and *aqp* expression), European eel pre-leptocephalus larvae reared at iso-osmotic salinity conditions seem to have a higher energy availability compared to larvae reared in full strength seawater (36 psu), which can then be utilized more efficiently to increase growth and survival.

Conclusion

To summarize, this study morphologically and molecularly elucidated osmoregulation related processes and together with the consideration of the most balanced mortality/deformity ratio, we conclude that salinity reduction benefits European eel larvae in terms of lower mortality and improved growth efficiency, which is likely facilitated by an energy surplus associated to lower osmoregulation demands. Hence, the overall knowledge gained from this study adds to our understanding of underlying biological mechanisms during ELH of European eel and provides a promising step towards optimized rearing conditions for European eel pre-leptocephalus larvae as well as in the strive for sustainable aquaculture of this species. Nevertheless, as the studied molecular parameters foreshadow an adequate subsequent larval development, likely due to a global lower osmoregulatory cost, further research is needed in order to verify the energetic developmental costs in contrasted salinity scenarios and evaluate the long-term morphological, molecular and physiological effects.

Supporting information

S1 File. Data.
(XLSX)

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Formal analysis: Sebastian N. Politis, Ian A. E. Butts.

Funding acquisition: Sebastian N. Politis, Jonna Tomkiewicz, Ian A. E. Butts.

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Visualization: Sebastian N. Politis.

Writing – original draft: Sebastian N. Politis.

Writing – review & editing: David Mazurais, Arianna Servili, Jose-Luis Zambonino-Infante, Joanna J. Miest, Jonna Tomkiewicz, Ian A. E. Butts.

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Study 5:

First-feeding by European eel larvae: A step towards closing the life cycle in captivity

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First-feeding by European eel larvae: A step towards closing the life cycle in captivity

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ABSTRACT

Breeding European eel in captivity is a complex task. However, recent advances in assisted reproduction and culturing techniques now allow mass production of high-quality gametes, embryos, and first-feeding larvae. Here, three studies were conducted to determine whether dietary regime, chemoattractants, and light intensity have an impact on the incidence of larval first-feeding, gut fullness, and behavior. Firstly, larvae at 12, 16, and 20 days post-hatch (DPH) were allowed to forage on one of five diets, rotifer paste (RP), RP + cod roe, RP + octopus juice, RP + live rotifers, or Sargasso Sea plankton for 2 h and afterwards evaluated for presence or absence of ingested diet. Secondly, light effects on feeding at 15 and 16 DPH were tested, using the following intensities: High light at 21.5 ± 3.9 μmol m^{-2} s^{-1}; intermediate at 6.8 ± 1.4 μmol m^{-2} s^{-1}; low at 0.6 ± 0.2 μmol m^{-2} s^{-1}; darkness; and a control where no feed was added in darkness. Lastly, behavioral observations were recorded on larvae at first-feeding. Results showed no evidence of first-feeding at 12 DPH. At 16 DPH, 23–50% of larvae had ingested feed when offered either RP, RP + live rotifers, or RP + cod roe. At 20 DPH, the highest incidence of feeding was detected when larvae were fed RP and chemoattractants; 8–30% of larvae ingested feed. Light had a positive impact on feeding at 15 and 16 DPH, such that incidence of first-feeding and gut fullness increased at the intermediate to high light intensities, where up to 50% of larvae ingested the RP diet and had 9% gut fullness. Swimming of larvae was characterized by bouts produced by undulations of the caudal region, followed by pauses where larvae remained motionless. Duration of swimming increased from 41 to 218 s at 13–17 DPH, respectively. Larvae exposed to RP-based diets swam more than the control (no feed). Larval attacks were observed in benthic and pelagic zones of the tank, however were more frequently observed in the benthos. Here, 8–10%, 60–73% and 69–86% of larvae were observed grazing on RP-based diets at 13, 15 and 17 DPH, respectively. Typically, attacks were associated with S-bend posture and upon initiation of S-bends the larvae remained stationary before an explosive lunge. At 13–17 DPH, larvae fed the RP-based diets exhibited a higher incidence of attacks than the control. Together, this work provides benchmark diets and conditions for future feeding and growth trials.

Statement of relevance: First-feeding by European eel larvae in captivity.

1. Introduction

European eel, *Anguilla anguilla*, is categorized as a critically endangered species (IUCN red list; Jacoby and Gollock, 2014) and has increasingly attracted scientific inquiry towards improving our knowledge relevant to species conservation and sustainable aquaculture. The spawning area of the European eel was delimited by Schmidt (1922) through the occurrence of early larval stages in the Sargasso Sea. Since then, researchers of this catadromous species have focused on solving the mystery of the eel life cycle, where most knowledge on reproduction is based on experimental studies (Palstra and van den Thillart, 2009). Early attempts reported success at establishing maturation, fertilization, as well as hatching embryos under laboratory conditions (Prokhorchik, 1986; Prokhorchik et al., 1987; Pedersen, 2004, Palstra and van den Thillart, 2009). European eel research has since progressed, enabling a steady production of "high-quality" gametes, embryos, and yolk-sac (pre-leptocephalus) larvae (Tomkiewicz, 2012; Mordenti et al., 2013; Butts et al., 2014, 2015; Sørensen et al., 2016a, 2016b). These advances have expanded the focus of experimental research to include larval performance in pre-feeding culture (Politis et al., 2014; Bouilliart et al., 2015; Sørensen et al., 2016b) and emerging first-feeding trials, leading to leptocephalus stages.

When initiating first-feeding, fish larvae detect prey via a wide range of chemical (olfaction and taste), visual, and mechanical stimuli (Rønnestad et al., 2013). For instance, natural or synthetic chemotactic stimulants attract larvae from a distance, foster the appropriate orientation, and promote the initiation of prey capture and ingestion (Kamstra and Heinsbroek, 1991; Reig et al., 2003; Barroso et al., 2013). In the case of Japanese eel, *Anguilla japonica*, larvae are attracted to and successfully

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feed on a slurry diet based on shark egg yolk (Tanaka et al., 2003), hen egg yolk or exoskeleton-free Antarctic krill (Okamura et al., 2013). Recently, rearing into glass eels was documented by feeding protein hydrolysate-based diets (Masuda et al., 2016), while a minute illoricate rotifer, *Proales similis* was tested as an alternative diet closer to natural larval trophic levels (Hagiwara et al., 2014). Unfortunately, suitable start feeds for larval European eel in culture have not been identified, while larval-prey interactions in nature still remain an enigma (Riemann et al., 2010), thus this area warrants immediate investigation.

In the Sargasso Sea, eel larvae are assumed to be present in the upper 100 m layer at night and migrate to greater depths with lower light conditions during daytime (Schoth and Tesch, 1984; Castonguay and McCleave, 1987). Such migration patterns have also been shown for cultivated Japanese eel (Otake et al., 1998). When reared in darkness, leptocephali still distributed near the surface at night and at the bottom during day, suggesting an endogenous circadian rhythm that may control the described vertical migration (Yamada et al., 2009). In nature, aquatic organisms may be exposed to highly variable light conditions, since several bio-physical factors as well as the water itself can alter the quantity and quality of incident light (Jerlov, 1968). Light intensity attenuates rapidly with depth, down to 0.5 lx at 200 m in the clearest ocean (Helvik and Walther, 1992) and has been shown to have a major effect on the highly susceptible early life history of fishes (Batty, 1987; Puvanendran and Brown, 1998). In culture, some species develop better in complete darkness like the Atlantic halibut, *Hippoglossus hippoglossus* (Bolla and Holmefjord, 1988) and gilthead sea bream, *Sparus aurata* (Sahin et al., 2001), or under constant light like haddock, *Melanogrammus aeglefinus* (Downing and Litvak, 1999) and Atlantic cod, *Gadus morhua* (Puvanendran and Brown, 2002a). For European eel, it has recently been shown that early life history stages are affected by light intensity, quality, and photoperiod, where highest survival to 5 days post-hatch (DPH) was detected under a 12 h light/dark, low intensity, red light photoperiod regime (Politis et al., 2014). This low light optimum coincides with the ontogeny of the larvae's feeding system during this early developmental window; i.e. mouth, pharyngeal opening, and eye development (Sørensen et al., 2016b).

The objective of this study was to test specifically tailored diets, under light and no-light conditions, for initiation of larval ingestion rate, gut fullness, and behavioral patterns. Our hope is that this work will provide a benchmark diet for further growth trials.

2. Materials and methods

2.1. Broodstock collection and husbandry

From Aug to Nov 2014 (at new moon), wild-caught female silver eel broodstock were obtained from a freshwater lake in northern Jutland, Denmark. Mean (±SEM) length and body weight of the females used in Studies 1 and 2 (see below) were 67.3 ± 3.5 cm and 521.3 ± 69.7 g, respectively ($n = 3$). After collection, broodstock were transferred to an experimental facility of the Technical University of Denmark (55.407444 N: 9.403414 E) where they were maintained in 300 L tanks equipped with a closed recirculation system at a max density of 10 females per tank under a continuous saltwater flow rate of 95 to 100 L min^{-1}. Additionally, in Nov 2015, wild-caught female silver eels (for Study 3 below) were obtained from Lough Neagh, Ireland (69.5 ± 0.4 cm and 673.0 ± 20.4 g, $n = 2$). Upon transport, they were all housed in a series of ~1250 L tanks equipped with a closed recirculation system. All males originated from a commercial eel farm (Stensgård Eel Farm A/S in Jutland, Denmark). Upon transport they were held in 300 L tanks equipped with a recirculating system at max density of 30 males per tank. Length and body weight of the males used in these studies were 39.6 ± 0.8 cm and 115.9 ± 8.7 g, respectively ($n = 17$). While in captivity, water temperature of the broodstock was maintained at ~20 °C and adjusted to ~36 psu using commercial sea salt.

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. To induce vitellogenesis females received weekly injections of pituitary extract at 18.75 mg kg^{-1} body weight (Argent Chemical Laboratories, Washington, USA; Kagawa et al., 2005; Tomkiewicz, 2012). To stimulate follicular maturation and induce ovulation, females received an additional injection of 17α,20ß-dihydroxy-4-pregnen-3-one (Sigma-Aldrich, St. Louis, MO, USA) at 2.0 mg kg^{-1} body weight (Ohta et al., 1996). Then, within 12–14 h, eggs were stripped from females. Males received weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich Chemie, Steinheim, Germany) at 150 IU per male (Gallego et al., 2012). Prior to fertilization, they were given another injection and milt was collected ~12 h after administration of hormone. Milt samples were pipetted into an immobilizing medium (Peñaranda et al., 2010) and used for fertilization within 4 h of collection.

2.2. Embryonic and larval rearing

For fertilizing and incubating eggs, North Sea natural seawater (~32.5 psu) was used (Sørensen et al., 2016a). Seawater was filtered using 0.8–1.0 μm cartridge filters (CUNO 3 M®, St. Paul, MN, USA) and then adjusted to 36 psu using Red Sea Salt (Red Sea International, Eilat, Israel).

For each female, diluted milt from several males was used. Milt dilution, gamete mixing, and activation were done according to Butts et al. (2014). After activation, newly fertilized eggs were transferred to 15 L of freshly filtered seawater (36 psu) for 2 h. Hereafter, only buoyant eggs were transferred to 60 L black conical flow though incubators that received filtered seawater at a flow rate of 30 mL min^{-1}. Eggs/embryos were kept in suspension by gentle aeration at 20.0 ± 0.5 °C. Each incubator was purged regularly to remove dead eggs.

After 45 h of incubation, the aeration was turned off, and the embryos started to initiate hatching. Following hatching, larvae were gently transferred (using 2–5 L beakers) to a matured water recirculating aquaculture system (RAS) that was equipped with (i) 4 × 250 L black larval rearing tanks, two 300 L seawater reservoirs, 0.45 m^3 biofilter filled with RK-bioelements (total biomedia-surface of 337 m^2–0.17 m^2 L^{-1} system water) and a ECO Runner 6000 protein skimmer (Aqua Medic, Bissendorg, Germany) (for Studies 1 and 2) or (ii) 30 × 50 L glass aquaria, 1800 L seawater reservoir, 600 L sump tank, two ECO Runner 3700 protein skimmers (Aqua Medic, Bissendorg, Germany) and a 40 W UV lamp (for Study 3). All larvae were reared at 19.0 ± 1.0 °C in 36 psu seawater (as above), under low light conditions (~0.3 μmol m^{-2} s^{-1} lx, Politis et al., 2014), with a constant flow rate of ~100 mL min^{-1}. Stocking density throughout the yolk-sac stage was between 20 and 50 larvae L^{-1}. Each rearing tank was siphoned, on a regular basis, to remove any bottom debris. Microalgae cells (*Nannochloropsis* sp.; 40,000–60,000 cells mL^{-1} of culture water; Reed Mariculture Inc., U.S.A.) were provided to the water to establish "green water" conditions for the duration of the experiment.

2.3. Study 1: effect of diet on ingestion rate

Larvae from 2 females were used to test the effect of diet on ingestion rate. In total, five diets were tested (Table 1). Larvae were randomly collected from the RAS system at 12, 16, and 20 DPH using a wide-mouth pipette (6 mm diameter); red light LED headlamps were used to assist with larval collection (Politis et al., 2014). Ten larvae were loaded into a series of 1 L glass beakers containing 800 mL of filtered 36 psu seawater (as above). Larvae were maintained inside temperature controlled cabinets (MIR-154, Sanyo, Panasonic Biomedical, Leicestershire, United Kingdom) at 20.0 ± 0.5 °C, under ~6.0 μmol m^{-2} s^{-1} light conditions. Each diet was tested in triplicate. The diets were added directly to the surface of each tank and they subsequently dispersed in the water

Table 1
Procedures used to manufacture, prepare, store, and deliver feed to first-feeding European eel, *Anguilla anguilla* larvae.

Feed source	Manufacturing and preparation	Storage and use	Feeding density
Rotifer paste	Rotifers (*Brachionus plicatilis*) were cultured in 200 L tanks on ORI-ONE[a] and RotiGrow Plus[b], according to company instructions. Instant algae,[c] (*Nannochloropsis oculata*) was provided daily at 40,000–60,000 cells mL^{-1}. Culture water was 0.2 μm filtered 32 psu seawater at 20 °C. At 100–400 rotifers mL^{-1} the cultures were harvested on a 40 μm screen and homogenized in a glass tube with a rotating pestle at 1200 rpm.[d] Homogenized rotifers were centrifuged at 8000 ×*g* for 5 min to remove excess fluid.	The precipitate was filled into disposable 1 mL syringes and frozen at −20 °C prior to use. Frozen rotifer syringes were thawed ~1 h prior to experimentation and added directly to feeding tanks.	Tanks received 0.5 mL of rotifer paste/L of water.
Rotifer paste + cod roe	Rotifer paste was prepared, as above. Freshly frozen Atlantic cod, *Gadus morhua* roe was thawed and liquids immediately sampled in Eppendorf tubes.	Cod roe liquid was frozen in tubes at −20 °C. Frozen rotifer syringes and tubes containing cod roe were thawed ~1 h prior to experimentation and added directly to feeding tanks.	Tanks received 0.5 mL of rotifer paste/L and 0.1 mL of cod roe liquid/L of water.
Rotifer paste + octopus juice	Rotifer paste was prepared, as above. Frozen wild-caught octopus, *Octopus vulgaris* was thawed and epidermal tissue parts grinded. Homogenate was filled in Eppendorf tubes and frozen.	Octopus liquid was frozen in Eppendorf tubes at −20 °C. Frozen rotifer syringes and tubes containing octopus juice were thawed ~1 h prior to experimentation and added directly to feeding tanks.	Tanks received 0.5 mL of rotifer paste/L and 0.1 mL of octopus juice/L of water.
Rotifer paste + live rotifers	Live rotifers were cultured and paste was prepared, as above.	Frozen rotifer syringes were thawed and live rotifers were harvested ~1 h prior to experimentation and added directly to feeding tanks.	Tanks received 0.5 mL of rotifer paste/L and 5 rotifers/mL of water.
Sargasso Sea plankton	Sargasso Sea plankton (avg. particle size was 3.35 ± 10.18 mm^2, $n = 141$ particles) was obtained from 16 March to 5 April 2014, between the longitudes 50°W and 68°W using a 25 m long conical net with mesh size of 560 μm and cod-end of 350 μm. The net was held open by a 3.5 m diameter steel ring. Fishing was performed using oblique hauls from surface down to 250 m, at a cruising speed of 2 knots and a total wire pay-out and retrieval of 55 min and 15 m min^{-1}, respectively.	Plankton samples were frozen at −20 °C immediately after catch. Frozen plankton was thawed ~1 h prior to experimentation and added directly to feeding tanks.	Tanks received 0.5 mL of plankton/L of water.

[a] ORI-ONE® (Skretting, Stavanger, Norway).
[b] Rotigrow Plus® (Reed Mariculture Inc., Campbell, CA).
[c] Nanno 3600 instant algae (Reed Mariculture Inc., Campbell, CA).
[d] Potter-Elvehjem PTFE 19 mm pestle with clearance of 0.101 mm–0.152 mm (Sigma-Aldrich Chemie, Steinheim, Germany.

column and sank to the bottom (benthic zone) within 60–90 s. Larvae were given an additional ~1 h acclimation period before each diet was added. The larvae were allowed to forage on the diets for ~2 h, upon which time they were removed from the glass beakers with a wide-mouth pipette and anaesthetized using tricaine methane sulphonate (MS-222, Sigma-Aldrich Chemie, Steinheim, Germany) at ~250 ppm. Afterwards, they were evaluated for the presence or absence of ingested diet. Images of larvae were then captured using a zoom stereomicroscope (SMZ1270i fitted DS-Fi2 Camera Head, Nikon Corporation, Tokyo, Japan). All images were analyzed for larval standard length (SL) and measurements were made using NIS-D software (NIS-Elements D, Nikon Corporation, Tokyo, Japan).

2.4. Study 2: effect of light intensity on the incidence of larval feeding and gut fullness

Light intensity effects on feeding were tested by applying the same quantity of rotifer paste (RP) that was found most successful in Study 1 (Table 1), to each of four replicate beakers each containing ten larvae. Larvae were randomly sampled from two batches of 15 DPH larvae and allowed to feed for ~2 h. The experiment was repeated the following day on a new aliquot sample of larvae at 16 DPH. The following five light intensity treatments were applied: High light at 21.5 ± 3.9 μmol m^{-2} s^{-1}; intermediate light at 6.8 ± 1.4 μmol m^{-2} s^{-1}; low light at 0.6 ± 0.2 μmol m^{-2} s^{-1}; darkness; and the control where the larvae received no feed and the trial was conducted in darkness. Light was applied using a daylight lamp (Falcon Eyes ML-40, 5400-5600K, Benèl BV in Hoogeveen, The Netherlands) and low light intensities were obtained by use of a shading garment. Each light intensity treatment was maintained inside temperature controlled cabinets at 20.0 ± 0.5 °C. After feeding, larvae were anaesthetized using MS-222 (as above) and photographed using a stereomicroscope (SMZ1270i fitted DS-Fi2 Camera Head, Nikon Corporation, Tokyo, Japan). All larvae were analyzed for SL, total gut area, and food particle(s) area. Gut fullness was also calculated as the area percentage of food particles relative to total gut area. All measurements were made using NIS-D software (NIS-Elements D, Nikon Corporation, Tokyo, Japan).

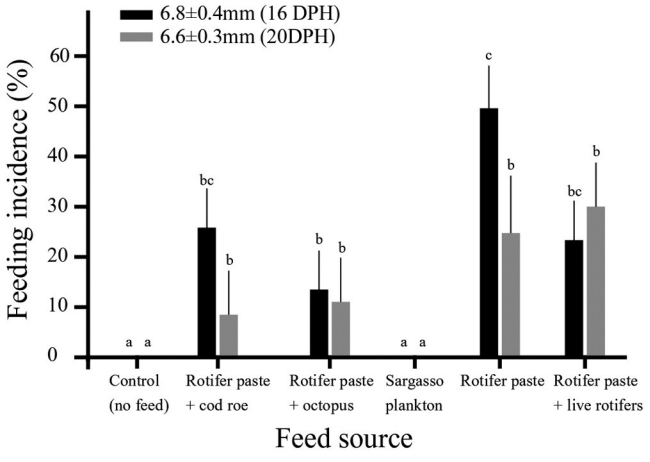

Fig. 1. Effect of dietary regime on incidence of larval feeding in European eel, *Anguilla anguilla* at 16 and 20 days post-hatch, with corresponding standard length. Diets with different letters are significantly different ($p < 0.05$, least square means, ANOVA). Error bars represent least square means standard error.

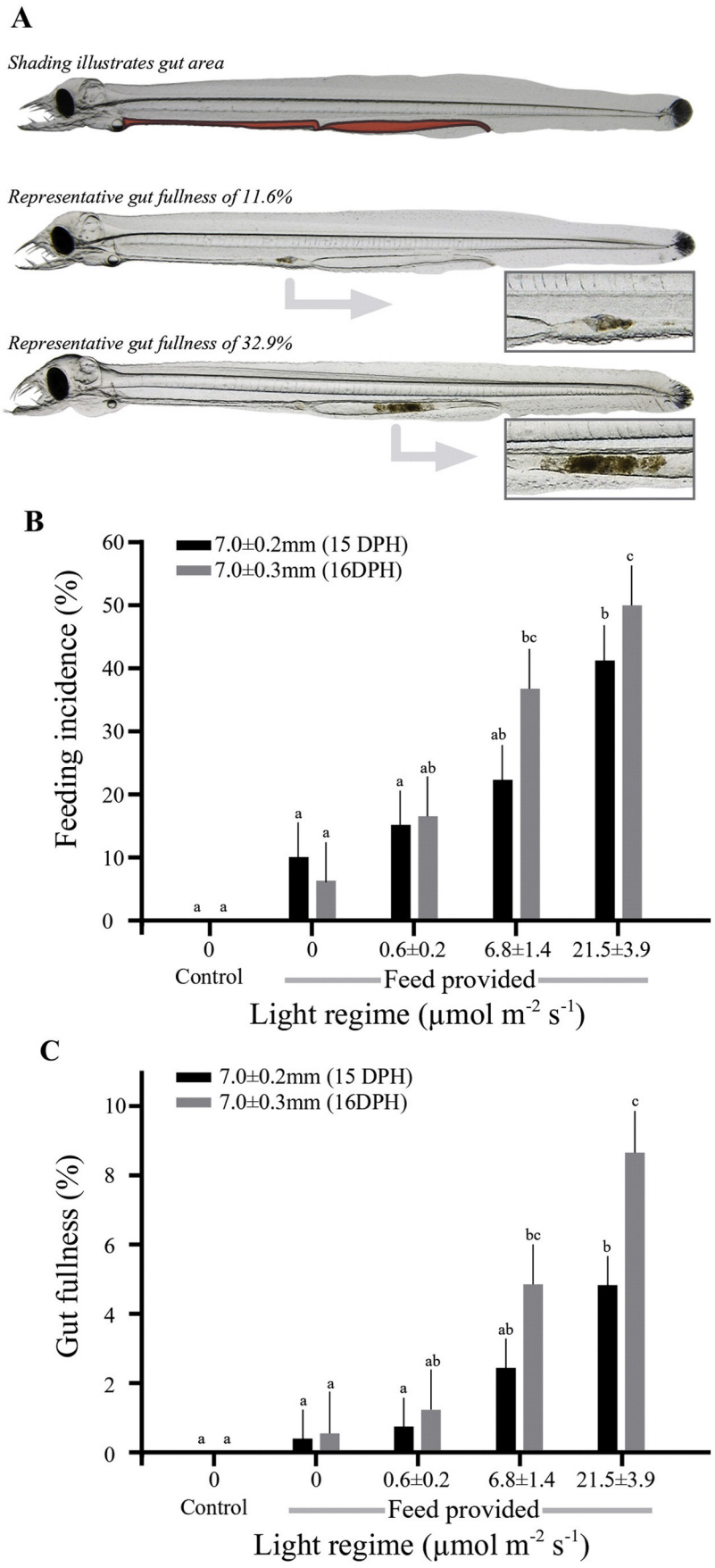

Fig. 2. (A) Illustration of European eel, *Anguilla anguilla* larvae with 11.6 or 32.9% gut fullness; (B) effect of light intensity on the incidence of larval feeding at 15 and 16 days post-hatch, with corresponding standard length; (C) effect of light intensity on the percentage of gut fullness at 15 and 16 days post-hatch, with corresponding standard length. Light intensities with different letters are significantly different ($p < 0.05$, least square means, ANOVA). Error bars represent least square means standard error.

2.5. Study 3: behavioral observations

Larvae from 2 females were used for behavioral observations at 13, 15 and 17 DPH. Firstly, larvae were arbitrarily selected from their respective rearing tanks and loaded into a series of 1 L glass beakers and subjected to high intensity light at 21.5 ± 3.9 μmol m^{-2} s^{-1} (according to Study 2). Fish were allowed a 3–5-min acclimation period before the appropriate dietary regime was added (RP, RP + live rotifers, RP + cod roe, or an experimental control that received no feed). Larval behavioral patterns were made by an observer seated quietly at eye level, ~30 cm in front of the beaker (Puvanendran and Brown, 2002b). Behavioral observations were conducted on 152 larvae (2 females × 3 dietary regimes + control × 3 ages × 5–8 larvae) using the focal-animal technique (Altman, 1974). For each trial, the following modal action patterns were recorded: time spent swimming (forward movement of larva through water column via tail undulations), pause (larva is motionless), percentage of time in benthic zone, attack (larva rapidly moves from stationary position with active jaw movement), and grazing (larva positioned in benthic zone with head submerged in feed particles). Following each trial, larvae were evaluated for the presence or absence of ingested diet (as above).

Additionally, feeding associated behavior was filmed using a SLR digital camera (20.2 megapixel EOS 6D, Canon, Ōta, Tokyo, Japan) equipped with 100 mm L f/2.8 macro lens (Canon). Filming was done perpendicular to the water surface in a culture tank (100 cm × 100 cm × 2000 cm). Camera settings were set at 25 frames s^{-1} at a resolution of 1980 × 1080 pixels pixels (1:1 ratio) using high compression (interframe, IPB), auto ISO, shutter speed of 1/50 s and f5.6. The tank was filled with natural sea water adjusted to 36 psu. RP was provided to a batch of larvae at 19 DPH. During feeding, tank surface light was kept at an intensity of ~5 μmol m^{-2} s^{-1}. Here, we monitored bouts of routine swimming, as well as S-bends and attacks. Video sequences of larval postures were isolated using Adobe Premier Pro CS5.5, Photoshop CS6 and InDesign CS6 (Adobe Systems Inc. San Jose, CA, USA).

2.6. Statistical analyses

Data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Data were arcsin square-root or $\log_{10}$ transformed when necessary (Zar, 1996). Alpha was set at 0.05. Tukey's honest significance test was used to determine which treatment means differed. For Study 1 and Study 2, ingestion rate and gut fullness data were analyzed using a series of one-way ANOVA models at each DPH. Here, the glass beaker was considered the replicate. For Study 3, time spent swimming, the percentage of time in benthic zone, and frequency of attacks were analyzed using a series of two-way repeated measures ANOVAs containing the time (13, 15, and 17 DPH; repeated factor) and dietary regime main effects, as well as the time × dietary regime interaction. When interactions were detected or suspected, reduced models were run separately at each level of time to facilitate their interpretation. Female was incorporated into the model as a random blocking factor and an individual larva was considered the replicate. Values are expressed as mean ± SEM.

3. Results

3.1. Study 1: effect of diet on ingestion rate

No evidence of first-feeding was observed at 12 DPH. However, at 16 DPH diet composition had a significant impact on larval feeding, such that larvae fed either RP, RP + live rotifers, or RP + cod roe exhibited the highest incidence of feeding, where 23–50% of larvae ingested these diets (Fig. 1). Dietary regime also had an impact on larval first-feeding at 20 DPH ($P < 0.05$). Here, diets formulated with RP, and associated chemotactic stimulants, gave the highest incidence of larval feeding (3 to 30%; Fig. 1). Larvae did not ingest the Sargasso Sea plankton diet nor were any feeding incidences detected for the control group, on either sampling day (Fig. 1).

3.2. Study 2: effect of light intensity on the incidence of larval feeding and gut fullness

For visualization, Fig. 2A depicts larvae with either 11.6 or 32.9% gut fullness. Lighting conditions had a significant impact on the incidence of larval first-feeding (Fig. 2B) and gut fullness (Fig. 2C) at 15 and 16 DPH. More specifically, the incidence of first-feeding and percentage of gut fullness increased in the intermediate (6.8 ± 1.4 μmol m^{-2} s^{-1}) and high (21.5 ± 3.9 μmol m^{-2} s^{-1}) light intensities (Fig. 2). In the high light intensity treatment, up to 49.9% of larvae had ingested the RP diet.

3.3. Study 3: effect of diet on larval behavior

Swimming was typically characterized by bouts produced by undulations of the caudal region, followed by pauses where the larvae remained motionless (Fig. 4A). Time and dietary regime had an impact on larval swimming ($P < 0.05$), where the duration of swimming significantly increased from 41.2 to 218 s at 13 to 17 DPH, respectively (Fig. 3A). Dietary regime also affected total swimming duration, such that swimming increased when larvae were exposed to RP + live rotifers or RP + cod roe, compared to the control ($P < 0.05$, Fig. 3B). Throughout the behavioral trials, larvae were typically detected in both benthic and pelagic zones. The percentage of time spent in the benthic zone was not impacted by either time, dietary regime, or the time × dietary regime interaction ($P > 0.05$).

Larval attacks were also observed in both zones; however, this modal action pattern was more frequently observed as grazing in the benthic zone where the majority of feed settled. For instance, 8–10%, 60–73%, and 69–86% of larvae were observed grazing on RP-based diets at 13, 15, and 17 DPH, respectively. On the contrary, no evidence of grazing behavior was observed for the control. Attacks were primarily associated with S-bend posture as outlined in Fig. 4A. Upon initiation of a S-bend the larva remained stationary using frequent caudal beats, with the posterior portion of their body, before an explosive lunge as visualized in Fig. 4A. The time × dietary regime interaction had a significant impact on the frequency of larval attacks. As a result, reduced models were run separately at each time to facilitate their interpretation. From 13 to 17 DPH, larvae fed RP-based diets typically exhibited a higher incidence of attacks than the control ($P < 0.05$, Fig. 4B).

At 13 DPH, 10% of larvae ingested the RP diet, while RP + live rotifers or RP + cod roe was not ingested. At 15 DPH, 27% (RP + cod roe) to 40% (RP) of larvae ingested diets, while at 17 DPH, 16% (RP + live rotifers) to 44% (RP) ingested diets. No evidence of first-feeding was observed for the control group.

4. Discussion

At present, experience in European eel larviculture is limited. In this study, we document the first evidence of first-feeding European eel larvae that have been reared in captivity. More specifically, up to 50% of cultured larvae ingested a diet composed of concentrated RP, with or without natural feeding stimulants. We also detected improved ingestion at higher light intensities. As such, our results move us a step closer towards understanding an undisclosed phase in the European eel life cycle; i.e. transition from newly hatched endogenous feeding larvae (pre-leptocephalus) to the exogenous leptocephalus stage.

In recent time, the life cycle for Japanese eel has been closed in captivity (Tanaka et al., 2003). In early larval feeding trials, Japanese eel larvae were offered and ingested S-type rotifers, *Brachionus rotundiformis* (Tanaka et al., 1995), and it was shown that they are able to absorb rotifer proteins in their intestine (Kurokawa et al., 1995). Since then, more

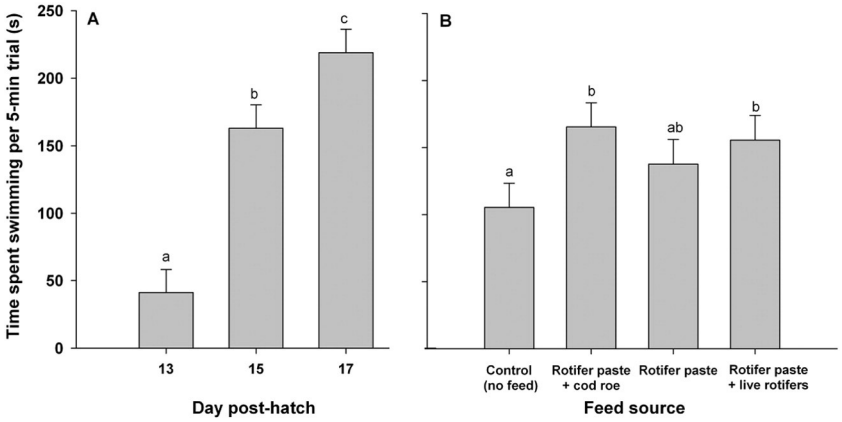

Fig. 3. Impact of larval age (A) and feed source (B) on swimming duration (per 5-min behavior trial) of European eel, *Anguilla anguilla* larvae. Treatments with different letters are significantly different ($p < 0.05$, least square means, ANOVA). Error bars represent least square means standard error.

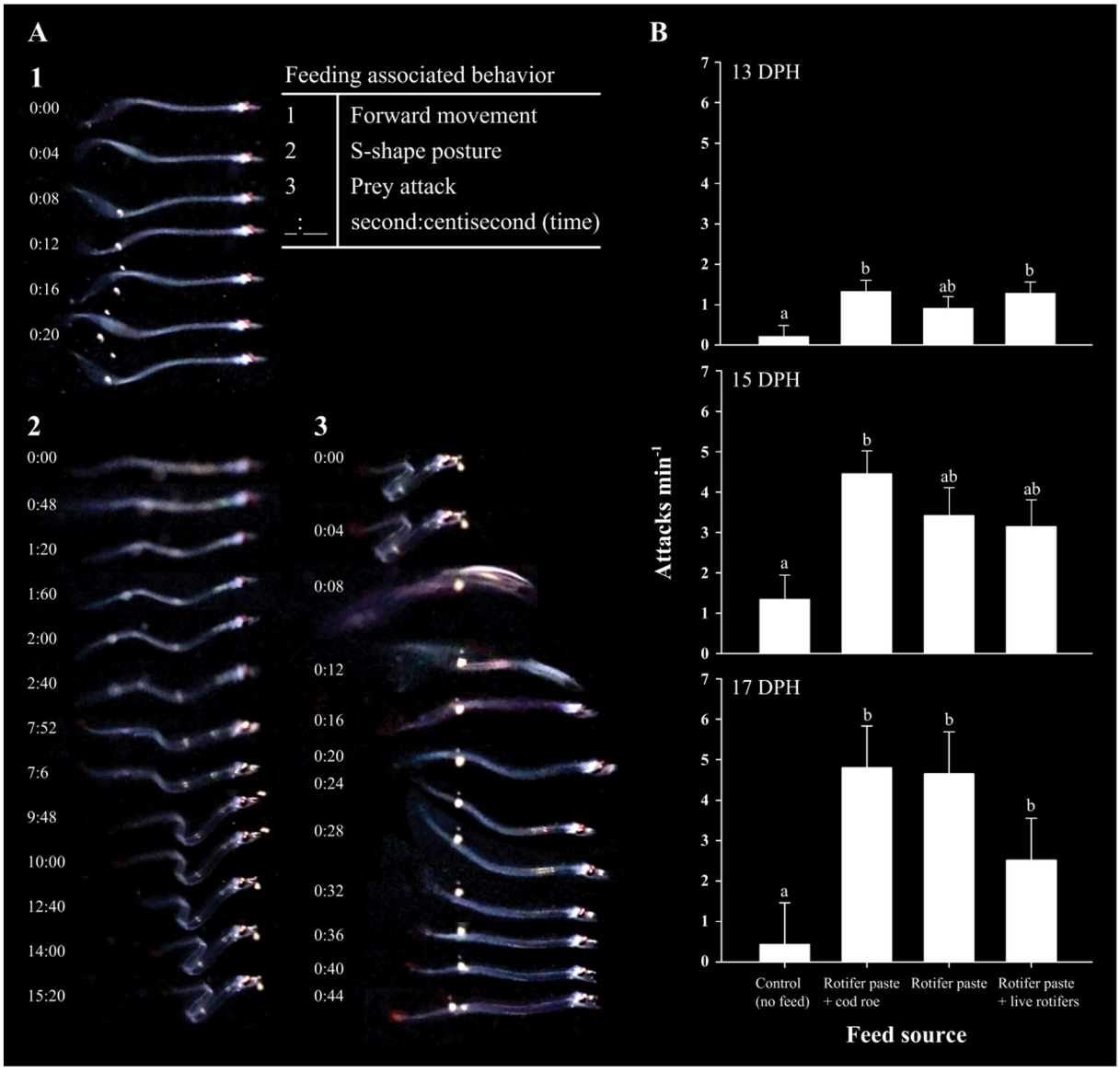

Fig. 4. (A) Illustrations of European eel, *Anguilla anguilla* feeding associated behaviors; (B) effect of feed source on the frequency of larval attacks at 13 to 17 days post-hatch. Feed sources with different letters are significantly different ($p < 0.05$, least square means, ANOVA). Error bars represent least square means standard error.

successful diets have been developed, where larvae can grow on a slurry diet based on shark egg yolk (Tanaka et al., 2001, 2003; Kagawa et al., 2005). However, the use of this slurry diet is not sustainable, due to depletion of shark populations (Baum et al., 2003). As such, alternative nutritional sources were tested and results showed that diets composed of hen egg yolk, exoskeleton-free Antarctic krill (Okamura et al., 2013), non-living *P. similis* (Hagiwara et al., 2014) and protein hydrolysate-based diets (Masuda et al., 2016) were good alternatives to endangered shark eggs for Japanese eel larvae. In our study, we show that European eel larvae are able to ingest RP. Thus, moving away from unsustainable diets, i.e. shark egg paste, to a more suitable start feed, provides a logical step towards sustainable aquaculture of eels.

Most fish larvae are visual feeders and foraging behavior is tightly linked to light conditions (Blaxter, 1986). Each species and populations thereof have adapted to specific conditions of light quantity, quality, and photoperiodic alterations. For instance, larval haddock had significantly greater feeding success when exposed to blue (470 nm) light and an intermediate intensity of 1.8 μmol m^{-2} s^{-1} (Downing and Litvak, 2001), while European sea bass, *Dicentrarchus labrax*, larvae grew better when reared under 12:12 h light/dark blue light (Villamizar et al., 2009); conditions most comparable to those of their natural aquatic environment. Atlantic cod was shown to develop best under constant light conditions (Puvanendran and Brown, 2002a), though a reduction in light intensity during the late larval stages seems to enhance feeding efficiency and growth (Monk et al., 2006). Feeding was significantly reduced at lower and higher light intensities for haddock larvae (Downing and Litvak, 2001), while cod larvae continue to actively feed at low intensity light levels that are close to darkness (Vollset et al., 2011). Similarly, we observed feed intake of European eel larvae in light but also in darkness, indicating that this species, besides using visual cues like Japanese eel (Pedersen et al., 2003), may also be able to use a wide range of chemical (olfaction and taste buds) and mechanical stimuli to detect prey (reviewed in Rønnestad et al., 2013). Nevertheless, in comparison to larvae kept in the dark, larvae at the intermediate (6.8 ± 1.4 μmol m^{-2} s^{-1}) 1) to high intensities (21.5 ± 3.9 μmol m^{-2} s^{-1}) had improved feeding success. Thus, it appears advantageous to rear European eel larvae, during first-feeding, in higher light intensity environments so that prey detection and capture can be optimized during this "critical" window (Hjort, 1914).

In the present study, larval behavioral patterns were observed from 13 to 17 DPH. Here, the goal was not to extrapolate these results to a natural environment, but to characterize behavioral repertoires that may be further used to optimize feeding success for future hatchery production. Most notably, we observed highly distinctive modes of swimming from short-term bouts, slow steady-state cruising to quick lunges for either prey attacks or spontaneous escape behaviors. Overall, swimming activity increased over the duration of the experiment, co-varied with the frequency of attacks and increased in the presence of RP + live rotifers or RP + cod roe. Together, this indicates that eel larvae were able to sense the RP-based diets, especially with added chemoattraction.

The general nature of the attack sequence (see Fig. 4) clearly shows that first-feeding eel larval are able to execute a complex goal-oriented motor response (McElligott and O'Malley, 2005). This sequence was characterized by "classic" modal action patterns (i.e. swim, pause, s-shape posture, capture, miss, pass) that are typically exhibited by fish larvae, such as Atlantic cod (Puvanendran and Brown, 1998; Puvanendran and Brown, 2002b), witch flounder, *Glyptocephalus cynoglossus* (Rabe and Brown, 2001), European sea bass (Villamizar et al., 2011) and zebrafish, *Brachydanio rerio* (Budick and O'Malley, 2000; McElligott and O'Malley, 2005). The frequency of attacks increased from 13 to 15–17 DPH, however ingestion rates declined when the larvae were initially offered the diets at 20 DPH (Fig. 1). Thus, at this later stage in ontogeny, the larvae likely reached "irreversible starvation" or the "point of no return" (Blaxter and Hempel, 1963).

The cod roe or octopus juice chemoattractants most probably increased the awareness of food availability, though did not significantly improve ingestion rate or the frequency of attacks for first-feeding European eel. This was surprising, considering that the addition of feeding stimulants increased feed intake and growth rate of glass eels (Heinsbroek and Kreuger, 1992) as well as of other fish species (Kolokovski et al., 1997; Barroso et al., 2013). Overall, prey attacks were observed throughout the water column, for RP-based diets with or without chemoattractants, and less frequently for the controls. Moreover, grazing behavior was commonly observed in the benthic zone where the majority of feed settled. Thus, it appears that European eel larvae, as well as Japanese eel (Tanaka et al., 2001), are able to feed from the tank substrate. As such, shallower tanks or raceways may increase larval ingestion as well as feed encounter rates.

5. Conclusion and future insights

Now that European eel larvae are ingesting feed in captivity, the next step will be to establish a dietary composition and regime that ensures high growth rates and survival. Together, this will resolve one of the major 'bottlenecks' in the process of establishing on-growing larval culture throughout the leptocephalus stage and thus to reach the phase of transformation to glass eels. To achieve this, a series of studies should be conducted to enhance knowledge on dietary requirements of European eel larvae. This includes a thorough examination of the ontogenic development of digestive tract morphology and a comprehensive analysis of pancreatic and intestinal enzymatic activity, as well as their corresponding gene expression. The capacity of larvae to digest and absorb various nutrients should also be investigated via a technique called "force-feeding", whereby in vivo studies target digestibility and assimilation of key nutrients using radiolabeled dietary nutrients (Rust et al., 1993). Based on attained insights, an assortment of feeds (formulated or live) could be manufactured to ensure adequate nutrition throughout the larval stage. Together, this will move us closer towards completing the life cycle in captivity for this endangered and economically important species of fish.

Acknowledgements

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Study 6:

Nutritional condition and molecular ontogeny of first-feeding European eel larvae

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Nutritional condition and molecular ontogeny of first-feeding European eel larvae

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Abstract

Digestive system functionality of fish larvae relies on the onset of genetically pre-programmed and extrinsically influenced digestive functions. This study explored how algal supplementation (green-water) until 14 days post hatch (dph) and the ingestion of food [enriched rotifer (*Brachionus plicatilis*) paste] from 15 dph onwards affects molecular maturation and functionality of European eel larval ingestion and digestion mechanisms. For this, we linked larval biometrics to expression of genes relating to appetite [ghrelin (*ghrl*), cholecystokinin (*cck*)], food intake [proopiomelanocortin (*pomc*)], digestion [trypsin (*try*), triglyceride lipase (*tgl*), amylase (*amyl*)], energy metabolism [ATP synthase F0 subunit 6 (*atp6*), cytochrome-c-oxidase 1 (*cox1*)], growth [insulin-like growth factor (*igf1*)] and thyroid metabolism [thyroid hormone receptors (*thrαA*, *thrβB*)]. Additionally, we estimated larval nutritional status *via* nucleic acid analysis during transition from endogenous and throughout the exogenous feeding stage. Results showed appetite stimulators (*ghrl*) and inhibitors (*cck*) marking the beginning of the first-feeding window on 12 dph, but no benefit of larviculture in green-water was observed. Moreover, expression of genes relating to protein (*try*), lipid (*tgl*) and carbohydrate (*amyl*) hydrolysis revealed essential digestive processes occurring from 14 to 20 dph. On 16 dph, a molecular response to initiation of exogenous feeding was observed in the expression patterns of *pomc*, *atp6*, *cox1*, *igf1*, *thrαA* and *thrβB*. Additionally, we detected increased DNA contents, which coincided with increased RNA contents and greater body area, reflecting growth in feeding compared to non-feeding larvae. Thus, the here applied nutritional regime facilitated a short-term benefit, where feeding larvae were able to sustain growth and better condition than their non-feeding conspecifics. However, RNA:DNA ratios decreased from 12 dph onwards, indicating a generally low larval nutritional condition, probably leading to the point-of-no-return and subsequent irreversible mortality due to unsuccessful exogenous feeding. In conclusion, this study molecularly identified the first-feeding window in European eel and revealed that exogenous feeding success occurs concurrently with the onset of a broad array of enzymes and hormones, which are known to regulate molecular processes in feeding physiology. This knowledge constitutes essential information to develop efficient larval feeding strategies and will hopefully provide a promising step towards sustainable aquaculture of European eel.

Key words: *Anguilla anguilla*, ingestion, digestion, gene expression, RNA/DNA, aquaculture

1. Introduction

Eel (*Angilla spp*) is a targeted, high-value species for aquaculture in Asia and Europe. Unfortunately, eel farming is still a capture-based industry exclusively relying on wild-caught glass eels and thus the sustainability of this industry is challenged by the present critically low stock abundance of especially European (*A. anguilla*) eels (ICES 2017). Hence, it is urgently needed to further develop and establish captive breeding techniques and technologies for this critically endangered diadromous fish species. However, eels do not reproduce naturally in captivity due to complex hormonal control mechanisms that relate to their long migration to native oceanic spawning areas (Vidal et al., 2004). Such maturational barriers can be overcome through hormonally assisted reproduction, which led to the first reports of Japanese eel, *A. japonica* (Yamamoto and Yamauchi 1974) and *A. anguilla* (Bezdenezhnykh et al., 1983) offspring obtained from artificially matured fish, more than 30 years ago. Since then, extensive scientific inquiry has moved the field from individual efforts of reproductive failure towards a stable production of Japanese eel offspring (Tanaka et al., 2001). Advances in Japanese eel culture have formed the baseline for eel research, leading to improved assisted reproduction protocols for European eel (Pedersen 2004; Paalstra et al., 2005; Sørensen et al., 2016a). However, establishment of culture technology throughout the larval stage until metamorphosis is still challenged by lack of insights on the "critical" early life history stages and dietary requirements for the unique pre-leptocephalus larvae.

As such, research has been conducted to identify natural larval eel feeding resources and early hypotheses such as eel leptocephali absorbing dissolved organic carbon or feeding on larvacean and zooplankton fecal pellets have been developed (reviewed in Miller 2009). Thereafter, a study investigating gut contents of European eel larvae caught in the Sargasso Sea, revealed that even the smallest larvae feed on a variety of planktonic organisms and that gelatinous zooplankton could be of fundamental dietary importance (Riemann 2010). Subsequently, a study on both natural and laboratory-reared larvae of the Japanese eel, estimated that leptocephali most probably feed on particulate organic matter (POM) such as marine snow and discarded appendicularian houses containing bacteria, protozoans and other biological materials (Miller et al., 2012). However, in spite of this increasing knowledge on natural larval eel feeding ecology, most insights gained have focused on non-*anguillid* species or older *anguillid* leptocephali, beyond the first-feeding stage (Miller, 2009). Thus, the natural first-feeding regimes of *Anguilla* pre-leptocephali still remain an enigma.

Similarly, increased scientific inquiry has focused on identifying potential first-feeding diets for laboratory reared eel larvae in aquaculture, where the first exogenously feeding eel larvae was reported two decades ago (Tanaka et al., 1995). Shortly after, the transition from the pre-leptocephalus to the leptocephalus stage was achieved, when Japanese eel larvae were fed a diet based on shark egg powder (Tanaka et al., 2001). Subsequent modifications of this diet led to the first laboratory reared glass eel production (Tanaka et al., 2003). However, the unstable supply of the eggs of the spiny dogfish (*Squalus acanthias*) used as a natural resource basis to develop this diet, in combination with the "vulnerable" status of this species (Fordham et al., 2016), has moved focus to more sustainable alternatives. Promising alternative diets based on fish protein hydrolysate

that had been pre-digested with integral enzymes from frozen krill (Masuda et al., 2013), or hen egg yolk and exoskeleton-free (skinned) Antarctic krill (Okamura et al., 2013) have been reported, but still with lower success compared to the shark paste. Additionally, Japanese eel larvae were observed to feed on various minute zooplankton species, suggesting that rotifers (such as *Proales similis*) could be an alternative initial food source for eel larvae (Wullur et al., 2013). Unfortunately, identifying suitable feeds for larval European eel has been rather stagnant for several decades and only recently it was documented that artificially produced European eel pre-leptocephali successfully ingested a diet based on rotifers (*Brachionus plicatilis*) with or without natural chemo-attractants (Butts et al., 2016).

Now that European eel research has succeeded in producing larvae (*via* assisted reproduction), which are able to exogenously feed, the opportunity has emerged to elaborate our knowledge on the nutritional condition of individual larvae *via* nucleic acid (RNA/DNA) content analysis (Clemmesen, 1993) and to examine physiological mechanisms regulating feeding, digestion and growth. Hormones that regulate feeding include appetite stimulators (orexigenic factors) such as ghrelin and inhibitors (anorexigenic factors) such as cholecystokinin (Volkoff et al., 2010). During early life history, fish larvae undergo major morphological and molecular changes, where shortly after mouth opening and before first-feeding, it is possible to detect an increasing availability of digestive enzymes relating to protein, lipid and carbohydrate hydrolysis, suggesting that the onset of the molecular digestive potential is genetically pre-programmed and not only influenced by the initiation of exogenous feeding (Zambonino-Infante and Cahu 2001). Similar to other fish species, eel larvae lack a stomach, so they probably depend on pancreatic enzymes (such as trypsin, lipase and amylase) for the extracellular hydrolysis of food (Kurokawa et al., 2002; Pedersen et al., 2003). It has also been shown that a sub-optimum nutritional composition can retard the maturational process of digestive enzymes (Krogdahl and Sundby, 1999), indicating that fish larvae are not able to handle some dietary components due to unique digestive capacities (Cahu and Zambonino-Infante, 1995). However, the maturational processes of digestive capacities can be stopped or delayed, but also enhanced depending on the dietary composition (Zambonino-Infante and Cahu 2001). Furthermore, rearing larvae in the presence of algae (green-water) can trigger digestive enzyme production (Cahu et al., 1998), which in addition to the genetically pre-programmed enzyme synthesis can induce an early maturation of hydrolytic functions (Lazo et al., 2000).

This demonstrates the necessity of determining species-specific molecular digestive potential and timing in order to understand the distinct nutritional predisposition and the capacity for adaptation towards utilizing dietary components, which might not occur in the corresponding natural feeding regime. As such, here we reared European eel larvae with or without the presence of algae (*Nannochloropsis*, *Pavlova* and *Tetraselmis*) from 0 to 14 dph and with or without the presence of food (rotifer paste) from 15 to 24 dph. Thereafter, we measured larval biometrics, quantified individual nucleic acid (RNA/DNA) contents and followed the relative expression of genes relating to appetite [ghrelin (*ghrl*), cholecystokinin (*cck*)], food intake [proopiomelanocortin (*pomc*)], digestion [trypsin (*try*), triglyceride lipase (*tgl*), amylase (*amyl*)], energy metabolism [ATP synthase F0 subunit 6 (*atp6*), cytochrome-c-oxidase (*cox1*)], growth [insulin like growth factor (*igf1*)], and thyroid metabolism [thyroid hormone receptors (*thrαA*, *thrβB*)]. Hence, the objectives

of this study were to i) explore the development of the endocrine systems which regulate appetite, ingestion and digestion by targeted gene expression; ii) molecularly define the "first-feeding" window; iii) examine the potential benefit of "green-water" during endogenous feeding; and iv) investigate the effect of initiating exogenous feeding on larval biometry, nutritional condition and gene expression in European eel.

2. Materials and Methods

2.1. Broodstock maturation and husbandry

Broodstock was kept at the EEL-HATCH facility in Hirtshals (Denmark), where females were held in 2000 L tanks and males in 500 L tanks, equipped with a closed recirculation system, under a continuous flow rate of ~15 L min^{-1}. Light conditions were held at low intensity (~20 lux) and 12 h day/12 h night photoperiod. Acclimatization took place over two weeks, in order to reach a salinity of 36 psu and temperature of 20°C. At the onset of experiments, broodstock fishes were anaesthetized (ethyl p-aminobenzoate, 20 mg L^{-1}; Sigma-Aldrich Chemie, Steinheim, Germany) and tagged with a passive integrated transponder, while initial length and weight were recorded. Farmed male fish originated from a commercial eel farm (Stensgård Eel Farm A/S) in Jutland, Denmark. Here, mean (±SD) total length and body weight were 37.10 ± 2.2 cm and 97.6 ± 15.80 g, respectively (n = 21). Males were matured by weekly injection of human chorionic gonadotropin (hCG, Sigma–Aldrich Chemie, Steinheim, Germany; 150 IU per male). Wild-caught female broodstock were obtained in late autumn 2015 from the Lough Neagh lake in Northern Ireland and had a mean (±SD) length and weight of 76.3 ± 4.3 cm and 875.0 ± 132.8 g, respectively (n = 3). Female broodstock were matured *via* weekly injections of freeze-dried carp pituitary extract based on whole glands (CPE, Ducamar Spain S.L.U., Cantabria, Spain) at a dose of 18.75 mg kg^{-1} initial body weight. Final follicular maturation was induced using the maturation inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma–Aldrich Chemie, Steinheim, Germany).

2.2. Water treatment and conditioning

Three types of water treatment were applied – Artificial seawater (ASW) used for gamete activation; filtered seawater (FSW) used for broodstock systems and conditioned filtered seawater (CFSW) used for embryo incubation and larval rearing. ASW was prepared using filtered tab water (reverse osmosis, Vertex Puratek 100 gpd RO/DI, Vertex Technologies Inc., CA, USA) adjusted to 36 psu using Sea salt (Red Sea International, Eilat, Israel; Sørensen et al., 2016b). FSW was based on seawater (pipeline from Skagerak, Denmark) and treated through a stepwise filtering process, passing through i) a glass bead filter (AstralPool S.A. Barcelona, Spain, 0.86 m^2 filter area, grain size 1-1.2 mm) for coarse particle removal, then through ii) three 20" cartridge filter in declining steps of 10, 5 and 1 µm pore size and last through iii) a UV lamp (MR1-220PP, 220W, UltraAqua, Aalborg, Denmark). CFSW was prepared by supplying FSW to a water conditioning system, allowing maturation of the water (>3months) following the principle presented by Vadstein et al.,

1993 and Attramadal et al., 2012. Maturation was achieved by long retention time and steady level of nutrition at a low level in recirculation fitted 3 × 15 m^3 biofilters filled with RK-bioelements (total biomedia-surface of 2.5 mill m^2 ~ 78.1 m^2 per L system water) and reservoir for automatic adjustment of temperature and water conductivity. Here, salinity was adjusted to 36 psu using artificial sea salt (Blue Treasure Reef Sea salt, Qingdao Sea-Salt Aquarium Technology Co., Ltd, China).

2.3. Gamete production and embryonic rearing

Gamete production and handling followed procedures described in Butts et al., 2014 and Sørensen et al., 2016ab. Upon mixing of gametes, ASW was used for zygote activation ensuring a salinity of 36 psu and temperature of 20°C. Early embryos were incubated in 15 L of ASW for 1 h, from where the buoyant egg layer was gently moved into new 15L of ASW. At 2 hours post fertilization (hpf), buoyant eggs were transferred to 60 L conical egg incubators and supplied with CFSW at a flow through rate of ~350 mL min^{-1}. Gentle aeration was added after ~10 hpf while temperature was lowered to 18°C for better embryonic development (Politis et al., 2017). Light was kept at a low intensity of ~10 lux (Politis et al., 2014) and twice a day sinking dead eggs were purged from the bottom valve of each incubator. At ~48 hpf aeration was stopped and embryos hatched at ~56 hpf.

2.4. Study 1: Green water

Directly after hatch, larvae were stocked in 2 identical rearing units, i) a recirculation system containing an algae mix (green-water) at ~40.000 cells/mL or ii) a recirculation system with clear seawater and no-algae (control). The algae mix consisted of commercially available frozen *Nanocloropsis* (2-5 µm), *Pavlova* (5-6 µm) and *Tetraselmis* (10-14 µm) species, representing different size groups (BlueBiotech Int. Germany). Each rearing unit facilitated a sump reservoir of ~1 m^3, from where water entered 4 × 80 L wet/dry trickle filters filled with RK-bioelements (240 m^2 surface area 0.12 m^2 per L) and thereafter reentered the sump. Here, a protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic Gmbh, Bissendorf, Germany) was included for removal of waste protein. Each rearing unit was attached to 3 × 250L tanks, each representing one of the 3 experimental larval batches, where flow rates were kept at ~10 L min^{-1} of CFSW. Initial stocking density was in the range of ~5000 larvae of one batch in a 250 L tank containing the algae mix and ~5000 larvae of the corresponding batch in a 250L tank with clear water (no algae).

2.5. Study 2: First-feeding

For this study, larvae of the same experimental batches were reared (from 0 to 14 dph) as the above mentioned experimental control in an identical recirculation system with clear seawater (no-algae) at a flow rate of ~10 L min^{-1} of CFSW. Similarly, the rearing unit was attached to 3 × 250 L tanks, each representing one of the corresponding batches. On 14 dph, ~75 larvae (25 of each batch) were gently transferred to each of 36 acrylic 2 L flow through jars (drz400sm hank, Jug Desk Type,

Taipei, Taiwan). The CFSW was again filtered (0.2 µm cartridge filter, CUNO 3M®, St. Paul, MN, USA) and then pumped into the bottom of each jar at a flowrate of ~10 ml × min^{-1}. All jars were randomly arranged, temperature was kept at 18°C (Politis et al., 2017), while light regime was set to 12 light / 12 dark photoperiod and intensity of 21.5 ± 3.9 µmol m^{-2} s^{-1} (Butts et al., 2016). From 15 dph onwards, 18 experimental jars received no-food (control), while the other 18 jars were fed an enriched rotifer (*Brachionus plicatilis*) paste diet (Butts el al., 2016) twice a day. Each portion weighed 706.5 ± 89.0 mg with a dry matter content of 41.3 ± 3.8 mg (n=8). Composition of enrichment: 5% moisture, 56% proteins and 17% lipids as well as 37mg/g DW n-3 HUFA and >5 DHA/EPA (ORI-ONE®; Skretting, Norway).

2.6. Larval biometry

Here, ~15 larvae per batch (3×) of each treatment for study 1 (algae / control) were randomly sampled on 4, 8, 12 and 14 dph, while ~15 larvae per replicate (3×) and treatment for study 2 (food / control) were randomly sampled on 15, 16, 18, 20 and 22 dph. Larvae were anesthetized using MS-222 (Sigma Aldrich Chemie, Steinheim, Germany) and photographed using a zoom stereomicroscope (SMZ1270i fitted DS-Fi2 Camera Head, Nikon Corporation, Tokyo, Japan) for assessment of larval standard length, yolk-sac and oil drop area as well as total body area using the NIS-Elements D software (Nikon Corporation, Tokyo, Japan).

2.7. Gene expression

For this molecular analysis, ~30 larvae from each replicate were randomly sampled at 4, 8, 12 and 14 dph in study 1 (algae / control) and at 15, 16, 17, 18, 20 and 22 dph in study 2 (food / control). Those 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 (264 ± 15 ng $µl^{-1}$) and purity (260/280 = 2.13 ± 0.002, 230/260 = 2.23 ± 0.008) were determined by spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany) and normalized to a common concentration of 100 ng $µl^{-1}$ with HPLC water. From the resulting total RNA, 680 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 transcription [PerfeCta® DNase I Kit (Quantabio, Germany)].

The expression levels of 11 target and 2 reference genes were determined by quantitative real-time PCR (qRT-PCR), using specific primers. Primers were designed using primer 3 software v 0.4.0 (http://frodo.wi.mit.edu/primer3/) based on cDNA and predicted cDNA sequences available in Genbank databases (Table 1). All primers were designed for an amplification size ranging from 75 to 200 nucleotides. The elongation factor 1 α (*ef1α*) and 40S ribosomal S18 (*rps18*) genes were chosen as housekeeping genes since 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).

Expression of genes in each larval sample from 2 randomly selected replicates, of each treatment and larval age were analysed in two technical replicates of each gene using the qPCR Biomark™ HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips) as previously described (Miest et al., 2015). 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 µL cDNA per sample for 10 min at 95°C; 14 cycles: 15 s 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 and measured using the ΔΔ 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).

2.8. Nucleic acid analysis

For this analysis, ~10 larvae per batch (3×) of each treatment of study 1 (algae / no-algae) were randomly sampled at 12 and 14 dph, while ~10 larvae per replicate (3×) and treatment of study 2 (food / no-food) were randomly sampled on 16, 17, 18, 20, 22 and 24 dph. Larvae were immediately euthanized using MS-222 and frozen at -20°C. Thereafter individual larvae were homogenized using 250-300 µl of sodiumdodecyl sulfate Tris buffer (Tris 0.05; NaCl 0.1M; SDS 0.01%; EDTA 0.01; pH 8) and the tissue homogenates were treated using the method described by Malzahn et al., 2003. Subsequently, fluorescence-photometric measurements using a specific nucleic acid dye [Ethidium bromide (EB), 2.5mg·ml^{-1}] were used to determine RNA and DNA content. In brief, total nucleic acid fluorescence was measured using an aliquot (130 µl) of each sample after adding 25 µl of EB solution. After total fluorescence was measured, RNAse (Serva Ribonuclease A, from bovine pancreas) was used to digest all RNA for 30 minutes at 37°C before the remaining fluorescence of the DNA was measured, allowing for RNA fluorescence to be estimated by subtracting the DNA from the total fluorescence. By using a 16S and 23S ribosomal RNA standard (Boehringer Mannheim) and measuring the RNA related fluorescence, the mass of RNA was calculated from a calibration curve, while the amount of DNA was determined by applying a slope factor to the RNA standard curve for DNA being 2.2 times higher compared to the RNA concentration slope values to account for the difference in fluorescence between the two (Huwer et al., 2011).

2.9. Statistical analysis

All data were analyzed using SAS statistical software (version 9.1; SAS Institute Inc., Cary, North Carolina). Residuals were tested for normality using the Shapiro-Wilk test and homogeneity of variances was tested using a plot of residuals versus fit values (PROC GLOT, SAS Institute 2003). Data were $\log_{10}$ or arcsine square-root-transformed when data deviated from normality and/or homoscedasticity (Zar 1996). Statistical models were used to investigate effects of “green

water" and "first-feeding" on larval biometry, gene expression and nucleic acid (RNA/DNA) content. Here, we analyzed the data using a series of repeated measures mixed-model ANOVAs (PROC MIXED; SAS Institute 2003). Models contained treatment (algae / control or food / control) and age (4 to 14 dph or 15 to 24 dph) main effects as well as the treatment × age interaction. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate (Littell et al., 1996). Treatment and age were considered fixed, whereas larval batch (study 1) or replicate (study 2) was considered random. Tukey's post-hoc analyses were used to compare least-squares means between treatments.

2.10. Ethics statement

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental 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. Larvae of European eel were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222). All efforts were made to minimize animal handling and stress.

3. Results

3.1. Biometry

During the endogenous feeding period, larval standard length significantly ($p < 0.0001$) increased from 3.25 ± 0.05 on 0 dph to 7.16 ± 0.05 on 14 dph (Fig. 1B), while larval body area significantly ($p < 0.0001$) increased from 1.51 ± 0.05 on 0 dph to 3.79 ± 0.07 on 14 dph (Fig. 1F). Concurrently, larval oil drop area significantly ($p < 0.0001$) decreased from 0.103 ± 0.002 μm^2 on 0 dph to 0.013 ± 0.002 on 14 dph (Fig. 1I). However, rearing larvae in "green water" did not have any significant influence on these larval morphometrics (Fig. 1A, E, H).

During the transition to exogenous feeding, larval oil drop area further significantly ($p < 0.0001$) decreased from 0.011 ± 0.001 on 15 dph until it was fully utilized (Fig. 1J), while larval standard length significantly ($p < 0.0001$) decreased on 24 dph (Fig. 1C). However, initiation of first-feeding did not significantly alter these larval morphometrics (Fig. 1D, K). Moreover, a significant ($p < 0.0001$) age × treatment (food / no-food) interaction was observed for larval body area, revealing that fed larvae retained a greater body area on 18, 20, 22 and 24 dph compared to non-feeding larvae; however still in a decreasing trend (Fig. 1G).

3.2. Gene expression

Expression of genes relating to appetite (*ghrl*, *cck*) significantly ($p < 0.0001$) increased during early development especially on 12 and 14 dph (Fig. 2B, F), while beyond this point, their mRNA

levels remained statistically constant from 15 to 22 dph (Fig. 2C, G). Similarly, genes encoding digestive enzymes (*try*, *tgl*) significantly ($p < 0.0001$) increased during early development and peaked on 14 dph (Fig. 2J, N), except for *amyl* which remained statistically constant (Fig. 2R). Additionally, all digestion related genes (*try*, *tgl*, *amyl*), continued to be expressed in constant high levels (similar to the corresponding levels on 14 dph) until significantly ($p < 0.001$) dropping on 22 dph (Fig. 2K, O, S).

On the contrary, expression of genes relating to energy metabolism (*atp6*, *cox1*) remained statistically constant throughout development (from 4 to 22 dph), irrespective of whether larvae were reared with or without algae as well as with or without food (Fig. 3A-L). Moreover, the gene relating to food intake (*pomc*) significantly ($p < 0.0001$) increased during the endogenous feeding stage with highest values on 12 and 14 dph (Fig. 3N). Subsequently, *pomc* significantly ($p = 0.006$) peaked on 16 dph after the initiation of first-feeding and decreased again beyond that point (Fig. 3O). Moreover, the expression of *thrβB*, relating to thyroid hormone metabolism, significantly ($p = 0.004$) decreased during the endogenous period and similarly to *pomc* showed a significant ($p = 0.0003$) peak on 16 dph following the transition to exogenous feeding; and then decreased beyond that (Fig. 3W). Furthermore, expression of *thrαA* (thyroid hormone metabolism) and *igf1* (growth and development) increased during the endogenous period, reaching highest constant values already from 8 to 14 dph (Fig. 3R, Z) and even though they both showed an elevated (non-significant) expression on 16 dph during exogenous feeding, mRNA levels remained statistically constant from 15 to 22 dph (Fig. 3S, AA).

However, generally rearing larvae with algae or initiating first-feeding did not significantly alter gene expression compared to larvae reared with no algae or no food, respectively.

3.3. Nucleic acid analysis

The “green water” principal did not significantly affect any fluorimetrically measured nucleic acid content (Fig. 4A, E, H), but it was observed that RNA content significantly ($p < 0.001$) decreased, DNA significantly ($p = 0.015$) increased, while the RNA/DNA ratio significantly ($p = 0.001$) decreased from 12 to 14 dph (Fig. 4 B, F, I). On the contrary, larvae taking up “first-feeding” showed a constant significant ($p = 0.007$) increase in RNA content compared to non-feeding larvae (Fig. 4 D). Additionally, a significant ($p = 0.043$) age × treatment (food / no-food) interaction was observed, revealing a significantly ($p < 0.05$) increased DNA content in feeding larvae on 17 and 18 dph compared to non-feeding larvae (Fig. 4 G). However, the RNA/DNA ratio was not significantly elevated in fed larvae (Fig. 4 K). Furthermore, it was observed that RNA and RNA/DNA ratio significantly ($p < 0.0001$) decreased throughout development (from 16 to 24 dph) irrespectively of whether the larvae were feeding or not (Fig. 4 C, J).

4. Discussion

The nutritional requirements of fish larvae are species-specific and even differ across developmental stages within a species, mainly due to the major morphological and physiological changes during ontogeny (Holt, 2011). Comparable to most species of young marine fish (Govoni

et al., 1986), the digestive system of eel larvae is undeveloped at hatch and forms into a narrow and straight digestive tract, with liver and pancreas elongated anteriorly from the middle part of the digestive tract along the esophagus, while the anus opens posteriorly (Kurokawa et al., 1995). However, the stomach differentiates only after metamorphosis into the glass eel stage, suggesting that larval eel digestion depends on the enzymatic functionality of the pancreas and gut (Kurokawa and Pedersen 2003). During early larval digestive system development, the activity of most fish digestive enzymes is initiated before the transition from yolk-sac larvae to exogenous feeding and is thus linked to underlying genetic mechanisms (Zambonino-Infante and Cahu 2001). Considering that genes encoding digestive enzymes were expressed irrespective of exogenous food ingestion, it seems reasonable to assume that this mechanism is linked to an internal clock, which is under endocrine control. The mechanism regulating feeding procedures includes appetite stimulators (orexigenic factors) such as *ghrl* and inhibitors (anorexigenic factors) such as *cck* (Volkoff et al., 2010). Hence, the expression of genes such as *ghrl* and *cck*, which seem to be involved in the molecular regulation of fish larval nutrition, can reveal the transition to exogenous feeding (Kurokawa et al., 2004; Ping et al., 2014). In our study, both *cck* and *ghrl* were expressed at basic levels already on 4 dph and peaked at 12 dph, indicating the molecular ontogenetic start of the first-feeding window in European eel pre-leptocephalus larvae. However, 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. Interestingly though, *cck* mRNA levels were significantly elevated prior to the first-feeding stage on 8 dph compared to the basal levels on 4 dph, indicating the potential adaptive capacity towards an earlier maturation of the digestive function and pancreatic enzyme secretion.

An early maturation of hydrolytic functions can be induced by the presence of algae during larval rearing, as shown in several fish species (reviewed in Reitan et al., 1997) and for instance improved larval growth in red drum, *Sciaenops ocellatus* (Lazo et al., 2000). Similarly, it was shown that green-water during larval rearing, acts by triggering digestive enzyme production earlier than clear water in sea bass, *Dicentrarchus labrax* (Cahu et al., 1998). Nonetheless, we did not observe any significant benefit of green-water, neither morphologically nor molecularly, during European eel pre-leptocephalus rearing. This could potentially be due to non-native algae species used in this study, not naturally occurring in the spawning area of European eel (Sargasso Sea), or the inert state of algae used which might impede triggering the desired effect of earlier maturation in digestive functionality. However, besides providing a direct nutritional supply and an indirect stimulation of appetite or digestive function, the presence of algae can influence the bacterial community of the rearing water and aid the microbial gut priming in fish larvae (Vadstein, 1993; Skjermo and Vadstein, 1993; Støttrup et al., 1995). Thus, it is possible that the here applied green-water rearing technique influenced the bacterial flora of the water and the microbial gut colonization, facilitating an earlier and improved larval digestion potential; however this was outside the scope of our study. Application of supplements directing gut microbiota such as probiotics, have received increasing attention in aquaculture, as it has been suggested that they feature a protective action on the intestinal mucosal cells, stimulating the innate immune response and thus causing an elevated state of immuno-readiness in fish such as tilapia, *Oreochromis niloticus* (Standen et al., 2013). Similarly, it was shown that the dietary addition of lactic acid

bacteria (probiotics) benefitted fish larvae by facilitating increased larval growth and decreased developmental deformities during early ontogeny of sea bass (Lamari et al., 2013). In this regard, the impact of algal presence or other nutritional supplementation such as probiotics during European eel larval rearing needs to be addressed in future research.

Gaining knowledge regarding digestive physiology during larval development for fish species, which are of interest to aquaculture, is essential for identifying adequate feeding strategies leading to improved production of healthy offspring. As such, several studies have utilized the recent advances in molecular tool availability in order to explore the molecular digestive system functionality and capacity in fish species such as Atlantic halibut, *Hippoglossus hippoglossus* (Murray et al., 2006), Atlantic cod, *Gadus morhua* (Kortner et al., 2011), catfish jundia, *Rhamdia quelen* (Silveira et al., 2013), blunt snout bream, *Megalobrama amblycephala* (Ping et al., 2014), Atlantic salmon, *Salmo salar* (Sahlmann et al., 2015) and Senegalese sole, *Solea senegalensis* (Canada et al., 2017). Similarly, intensive scientific inquiry has been subjected to identify the molecular functionality and capacity of the digestive tract in Japanese eel larvae during the transition from endogenous to exogenous feeding, where it was demonstrated that expression levels of genes encoding the major pancreatic enzymes (such as trypsin, amylase and lipase), arise prior to or at initiation of exogenous feeding (Kurokawa et al., 2002; Pedersen et al., 2003; Murashita et al., 2013). In this study, we demonstrate the digestive function ontogeny of European eel larvae, *via* transcriptomics of selected genes encoding some of the most important digestive enzymes relating to protein, lipid and carbohydrate hydrolysis. All enzymes were detected at basal expression levels already on 4 dph and increased throughout ontogeny to reach peak values on 14 dph, corresponding to the period of increasing exogenous feeding incidences in this species (Butts et al., 2016). Moreover, we show that the transcript levels of protein (*try*) digestion enzymes were higher than those of carbohydrate (*amyl*) and lipid (*tgl*) digestion enzymes (Fig. 5A), similar to the findings for pre-leptocephali and leptocephali of Japanese eel (Hsu et al., 2015), indicating a nutritional predisposition for proteins during those life stages. This should also be in accordance with their natural feeding regime, as it is assumed that they feed on marine snow, primarily consisting of protein detritus (Miller et al., 2012). Similar to the Japanese eel findings (Hsu et al., 2015), we also detected elevated expression levels of *amyl* (carbohydrate hydrolysis) within the first-feeding window, which might reflect a primary mode of digestion (Zambonino-Infnate and Cahu, 2001), but it cannot be considered that eel pre-leptocephalus larvae have a predisposition towards utilizing carbohydrates as a main energy source.

In this study, European eel larvae were first fed (on 15 dph) a paste consisting of enriched rotifers (*Brachionus plicatilis*) as previously described in Butts et al., 2016. On 16 dph, a molecular response to initiation of exogenous feeding (Fig. 5B) was observed in the expression pattern of genes relating to energy metabolism (*atp6*, *cox1*), food intake (*pomc*), growth (*igf1*) and thyroid metabolism (*thrαA*, *thrβB*). This up-regulation was not observed in non-feeding larvae and was purely driven by the expression profiles of those genes in the feeding treatments, but since it only occurred on this one time point, it was not sufficient to be detected by the applied statistical model. Nevertheless, as larvae ingested the diet resulting in similar gut fullness to previously reported findings (Butts et al., 2016), we conceive this as a temporary positive reaction to the ingestion of this exogenous diet. However, considering that this positive trend of up-regulated expression of

those genes vanished already on the consecutive day, it is clear that even though larvae successfully ingested the rotifer paste diet, it apparently did not comprise the appropriate nutritional value needed to sustain growth and survival during this critical developmental stage. Concurrently, the mRNA expression of all digestive enzymes remained at an elevated level from first-feeding and throughout ontogeny until dropping on 20 or 22 dph. This, combined with the observed degeneration of larval tissue (decreased body area) and the fact that no larvae survived beyond 30 dph, indicate the end of the window of opportunity for larvae to ingest and digest exogenous food and the transition into the point-of-no-return, where larvae that failed to successfully take up exogenous feeding and assimilate ingested nutrients into growth, enter a period of irreversible starvation.

In recent years, major progress has been achieved regarding molecular tools, improving the sensitivity of analytical methods, such as the application of fluorometric techniques to investigate RNA/DNA ratios at individual level of even small organisms such as fish larvae (Clemmesen, 1993; Clemmesen, 1994). The RNA/DNA ratio provides an indication of the protein-synthesizing potential of an organism and has been considered a valuable tool to be used as a biochemical indicator of the physiological and nutritional state as well as growth of aquatic organisms (Buckley et al., 2008; Chicaro and Chicaro 2008). The principal of the RNA/DNA ratio is based on the assumption that under changing conditions the amount of DNA is stable within the somatic cells of a given species (and at a given developmental stage), unless the amount of cells (growth or deterioration) is changing (Foley et al., 2016). Thus, DNA content can increase throughout development, since the DNA content per cell remains constant, but the total cell number increases with growth (Ferron and Leggett, 1994). In contrast to DNA, the amount of RNA varies with changing nutritional conditions as it directly drives gene expression and protein synthesis. Thus, a recently well-fed, metabolically active, growing individual should have a relatively high RNA:DNA ratio compared to a starving, metabolically inactive individual (Buckley et al., 1999). In our study, we observed a significantly higher amount of RNA in feeding compared to non-feeding larvae, throughout the entire investigated period, which is a clear indication of an increased metabolic activity associated to protein synthesis, as a direct response to initiation of exogenous feeding. Moreover, we observed an increased amount of total DNA in feeding compared to non-feeding European eel larvae on 17 and 18 dph, which in combination with the increased amount of RNA and greater body area observed, especially on 18 and 20 dph can be associated to growth in feeding and faster deterioration in starved larvae.

Considering fish RNA/DNA ratios, low values are commonly correlated to starvation (Chicaro and Chicaro, 2008). European eel larval RNA/DNA ratios in our study ranged only from 0.66 ± 0.01 on 12 dph to 0.40 ± 0.03 on 24 dph. However, similarly low values (<0.5) have been reported for American glass eels (Laflamme et al., 2012) and only slightly elevated (0.8-1.2) for Japanese glass eels (Kawakami et al., 1999); although none of these values can be directly compared. The relatively low RNA/DNA ratios could also indicate an eel specific developmental strategy, characterized by a generally low metabolic activity during this early life phase. During this migratory phase in nature, eel offspring probably down-regulate metabolic expenses in order to survive their oceanic journey, while efficiently drifting *via* oceanic currents (Castonguay and McCleave, 1987; McCleave et al., 1998). Nevertheless, in our study, the larvae reared with the

presence of algae and/or taking up first-feeding, did not show an improved RNA/DNA ratio, even if a time lag in response of a few days was taken into consideration (Peck et al., 2015). This is due to nucleic acid ratios providing a measure of growth and condition only within a recent time window (1-4 days) which depends on environmental factors such as temperature (Buckley et al., 1999; Clemmesen, 1994). Actually, the here measured RNA/DNA ratio as well as the RNA content per larva constantly decreased from 12 dph onwards, indicating a low larval nutritional condition and a lack of successful nutrient assimilation. Additionally, the positive trend of greater DNA amounts in feeding larvae only lasted for a short-term period and DNA content followed the decreasing pattern of larval body area, leading to the unavoidable point of no return and subsequent irreversible starvation due to unsuccessful utilization of exogenous feeding.

To summarize, we here explored the endocrine regulation of feeding, molecularly identified the "first-feeding window" and digestion potential as well as investigated the larval nutritional status and molecular response to green water and first-feeding during the transition from endogenous to exogenous feeding in European eel larvae. Thus, this study has demonstrated sensitive indicators of nutritional and molecular aspects around first-feeding. Together, this will help to better define feeding strategies during larviculture of this species, including the appropriate choice of nutrient sources, that will facilitate the digestive tract ontogeny and functionality as well as hopefully lead to improved growth and survival towards metamorphosis. In conclusion, the here applied nutritional regime facilitated a short-term benefit, where feeding European eel larvae were able to sustain growth and better condition than their non-feeding conspecifics. Even though a long-term advantage was not achieved, the knowledge gained provides a great step towards closing the life cycle in captivity and will hopefully provide a promising step towards sustainable aquaculture of this species.

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Table 1: Sequences of European eel (*Anguilla anguilla*) primers used for amplification of genes by qRT-PCR. Primers were designed from cDNA or predicted cDNA (*) sequences available in Genbank databases or from loci annotation in European eel (Eel loci). The European eel genome was obtained from zf-genomics (Henkel et al., 2012). The table also lists function, corresponding database and accession number of target gene sequences, as well as position of the primer on the referenced sequence.

Full name	Abbrev.	Function	Database	Accession Number	Primer sequence (5' 3') (F: Forward; R: Reverse)
Prepro-Ghrelin*	*ghrl*	Appetite	GenBank Nucleotide	AZBK01848791	F: CCCACTGTGAGCTTCAGACA
					R: TGGACAGAGTCCATCCACAG
Cholecystokinin*	*cck*	Appetite	GenBank Nucleotide	AZBK01795176	F: CGCCAACCACAGAATAAAGG
					R: ATTCGTATTCCTCGGCACTG
Trypsin	*try*	Digestion	GenBank Nucleotide	MH001533	F: TGCAGATCAAGCTCAGCAAG
					R: ATCGTTGGAGCTCATGGTGT
Triglyceride lipase	*tgl*	Digestion	GenBank Nucleotide	DQ493916	F: CTGACTGGGACAATGAGCGT
					R: CGTCTCGGTGTCGATGTAGG
Amylase*	*amyl*	Digestion	Eel loci	g472	F: AGACCAACAGCGGTGAAATC
					R: TGCACGTTCAAGTCCAAGAG
ATP synthase F0 subunit 6	*atp6*	Energy metabolism	GenBank Nucleotide	NC_006531	F: GGCCTGCTCCCATACACATT
					R: GACTGGTGTTCCTTCTGGCA
Cytochrome-C-Oxidase	*cox1*	Energy metabolism	GenBank Nucleotide	NC_006531	F: CTACTCCTCTCCCTGCCAGT
					R: CTTCTGGGTGGCCGAAGAAT
Proopiomelanocortin	*pomc*	Food intake	GenBank Nucleotide	JX441983	F: GCCTGTGCAAGTCTGAACTG
					R: GACACCATAGGGAGCAGGAA
Insulin like growth factor 1	*igf1*	Growth	GenBank Nucleotide	EU018410	F: TTCCTCTTAGCTGGGCTTTG
					R: AGCACCAGAGAGAGGGTGTG
Thyroid Hormone Receptor α A	*thrαA*	Thyroid metabolism	GenBank Nucleotide	KY082904	F: GCAGTTCAACCTGGACGACT
					R: CCTGGCACTTCTCGATCTTC
Thyroid Hormone Receptor β B	*thrβB*	Thyroid metabolism	GenBank Nucleotide	KY082907	F: GAAGACTGAGCCCTGAGGTG
					R: AGGTAATGCAGCGGTAATGG
Elongation Factor 1 α	*ef1a*	Housekeeping	GenBank Nucleotide	EU407824	F: CTGAAGCCTGGTATGGTGGT
					R: CATGGTGCATTTCCACAGAC
40S Ribosomal S18	*rps18*	Housekeeping	GenBank TSA	GBXM01005349	F: TGACCGATGATGAGGTTGAG
					R: GTTTGTTGTCCAGACCGTTG

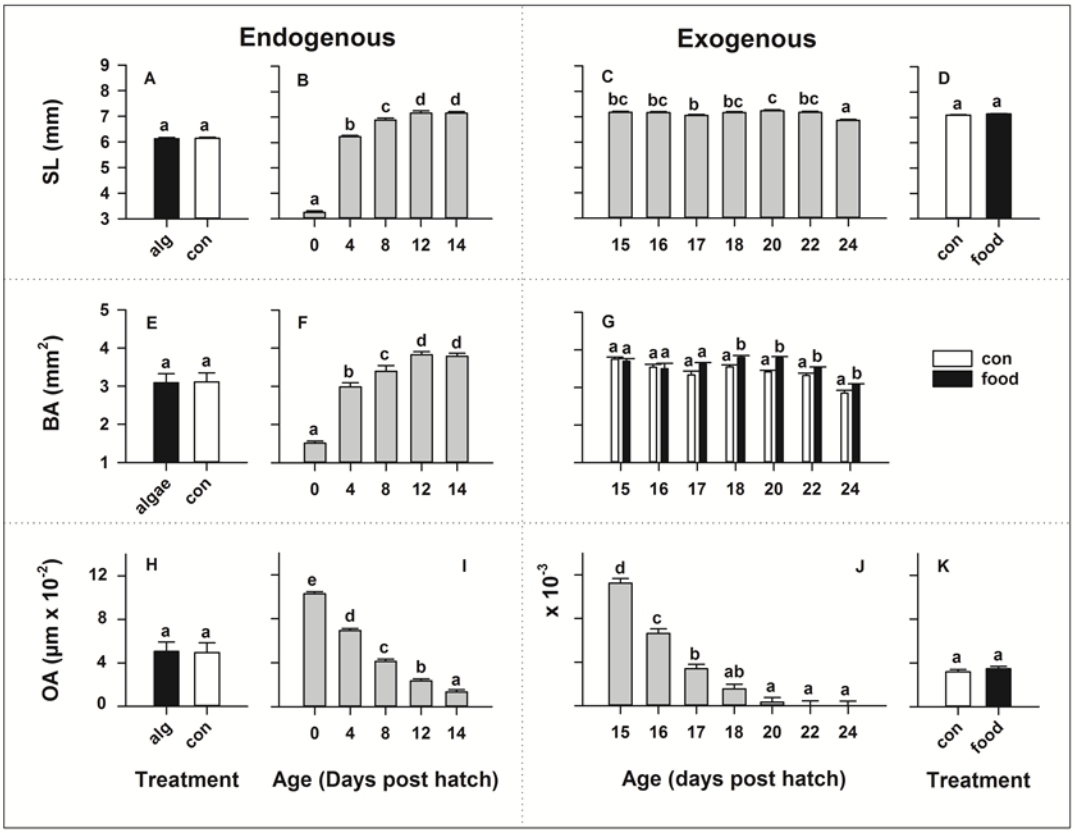

Figure 1: European eel (*Anguillla anguilla*) larval biometrics and the effect of green-water [algae (alg) *vs* no-algae (con)] during endogenous feeding and of first-feeding [food *vs* no-food (con)] during exogenous feeding. Standard length (A-D), body area (E-G) and oildrop area (H-K). Values represent means (± SEM) among three crosses at each age and treatment. Lower case letters represent significant differences ($p < 0.05$).

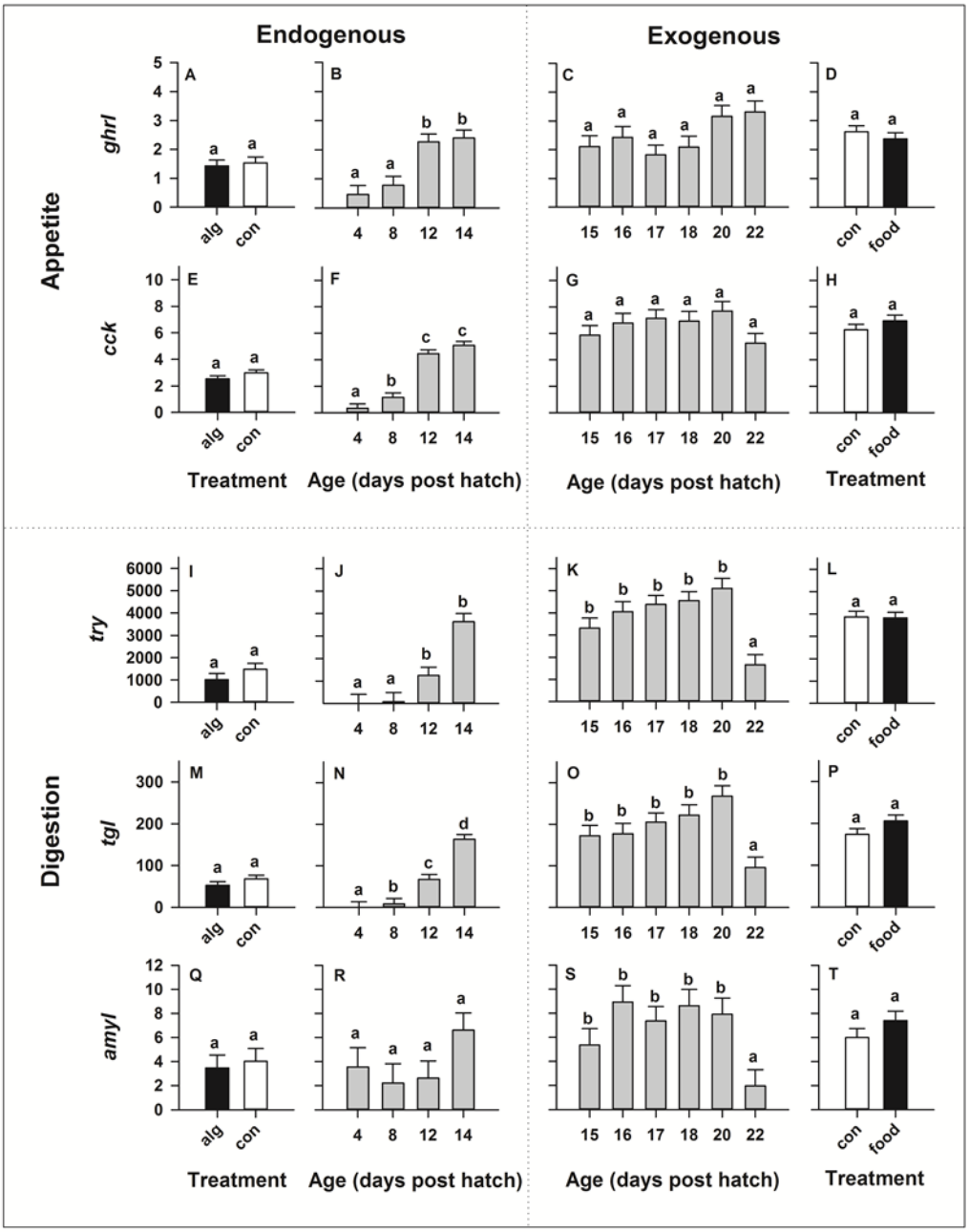

Figure 2: European eel (*Anguillla anguilla*) larval relative gene expression and the effect of green-water [algae (alg) *vs* no-algae (con)] during endogenous feeding or first-feeding [food *vs* no-food (con)] during exogenous feeding. Relative expression of the appetite related orexigenic ghrelin (*ghrl*: A-D) and anorexigenic cholecystokinin (*cck*: E-H) as well as relative expression of genes encoding digestive enzymes relating to protein [trypsin (*try*): I-L], lipid [triclyceride lipase (*tgl*): M-P] and carbohydrate [amylase (*amyl*): Q-T] hydrolysis. Values represent means (± SEM) among three crosses at each age and treatment. Lower case letters represent significant differences ($p < 0.05$).

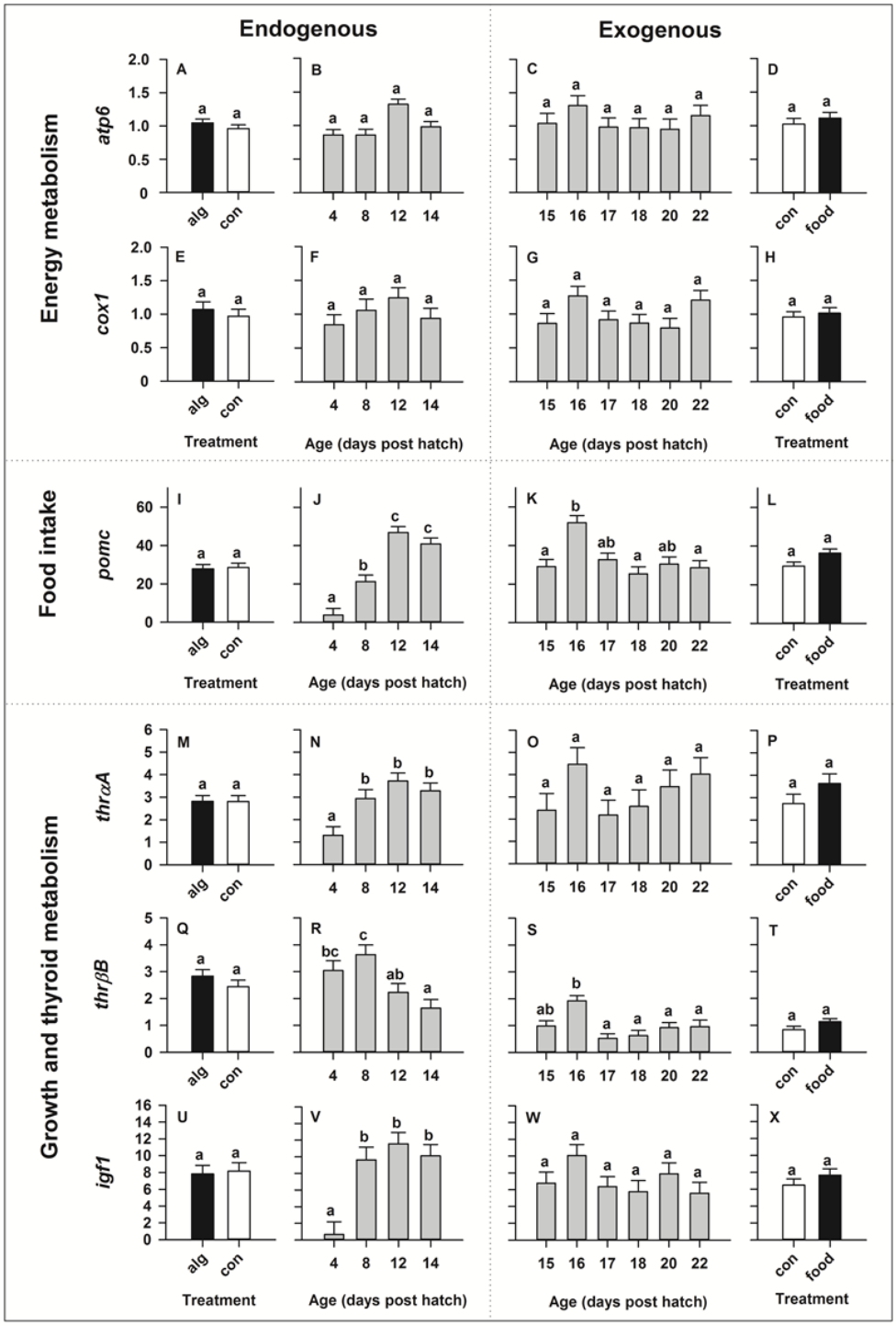

Figure 3: European eel (*Anguillla anguilla*) larval targeted gene expression and the effect of green-water [algae (alg) *vs* no-algae (con)] during endogenous feeding or first-feeding [food *vs* no-food (con)] during exogenous feeding. Relative expression of genes relating to energy metabolism [ATP-synthase-F0-subunit-6 (*atp6*: A-D), cytochrome-c-oxidase (*cox1*: E-H)], food intake [proopiomelanocortin (*pomc*: I-L)], thyroid metabolism [thyroid-hormone-receptors (*thrαA*: M-P and *thrβB*: Q-T)] and growth [insulin-like-growth-factor-1 (*igf1*); U-X]. Values represent means (± SEM) among three crosses at each age and treatment. Lower case letters represent significant differences ($p < 0.05$).

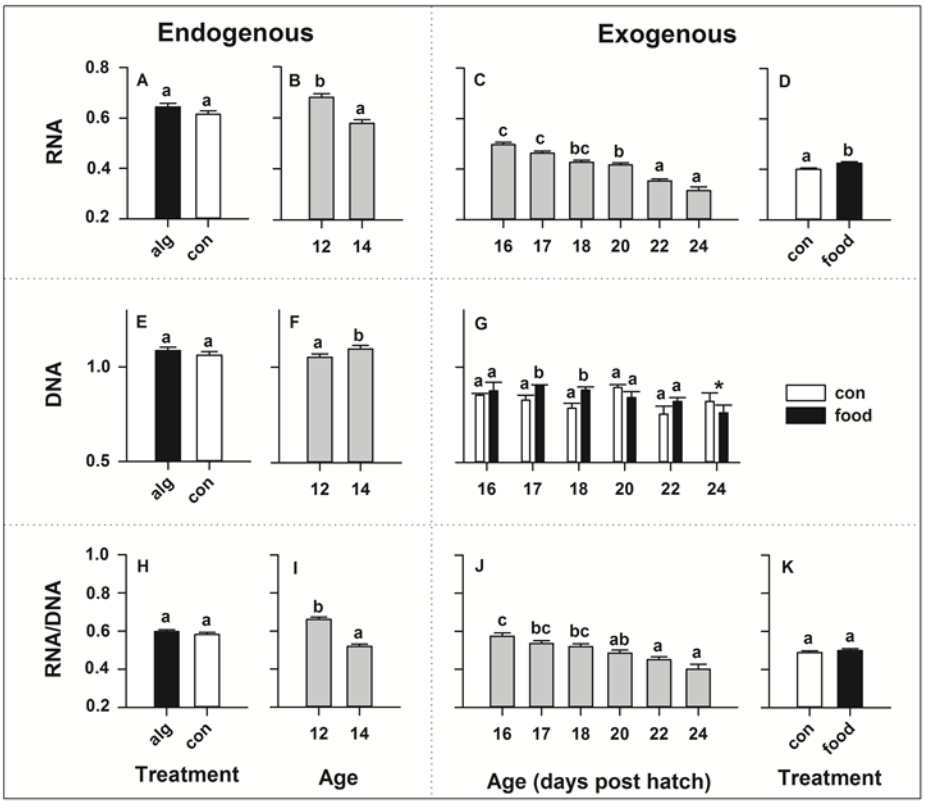

Figure 4: European eel (*Anguillla anguilla*) individual larval nucleic acid content and the effect of green-water [algae (alg) *vs* no-algae (con)] during endogenous feeding or first-feeding [food *vs* no-food (con)] during exogenous feeding. Total RNA (A-D) or DNA (E-G) content and RNA:DNA ratio (H-K). Values represent means (± SEM) among 5-10 individual larvae from 3 replicates at each age and treatment. Lower case letters represent significant differences ($p < 0.05$).

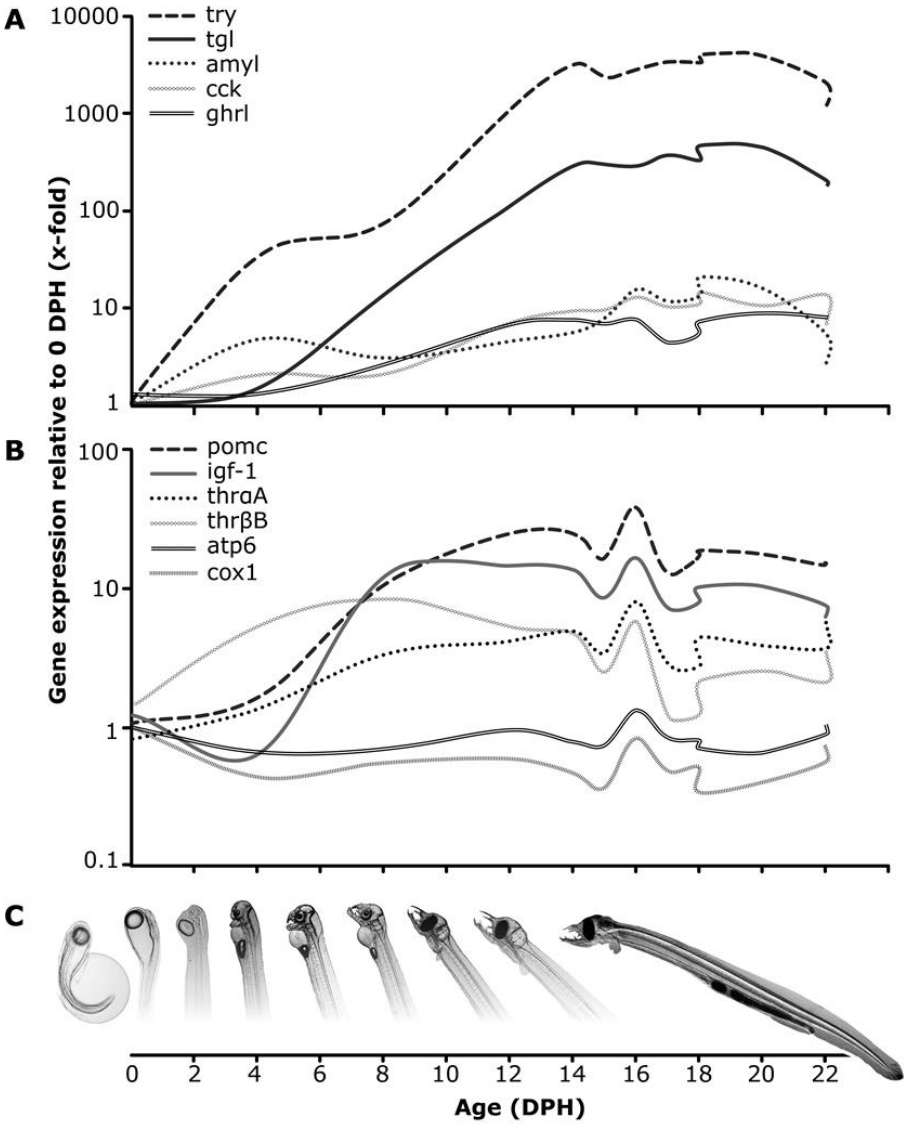

Figure 5: Conceptual overview - Expression ($2^{-\Delta\Delta ct}$) was calculated in relation to the average expression on 0 days post hatch of each gene. **A:** Relative expression for trypsin (*try*), triglyceride lipase (*tgl*), amylase (*amyl*), cholecystokinin (*cck*) and ghrelin (*ghrl*); **B:** Relative expression for proopiomelanocortin (*pomc*), insulin-like growth factor (*igf1*), thyroid hormone receptors (*thrαA*, *thrβB*), ATP synthase F0 subunit 6 (*atp6*) and cytochrome-c-oxidase (*cox1*); **C:** European eel pre-leptocephalus larval development from hatch until the feeding stage.

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The End

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