

Developmental timing of key molecular mechanisms and applicability of salinity reduction regimes for European eel larval rearing

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Abstract

There is an urgent need to close the life cycle and develop sustainable aquaculture for European eel. Increased scientific inquiry focuses on developing rearing techniques as natural conditions cannot be accurately consulted. Eel offspring naturally occur in the ocean, but reducing salinity towards iso-osmotic conditions benefits larval performance. This study tested salinity reduction scenarios during eel larvi-culture, using recirculating aquaculture systems. Larvae from different families were reared at constant 36 psu (experimental control) or introduced to a salinity reduction (36 to 18 psu) initiated on 3 days post hatch (dph), at a rate of 4 psu per day, occurring within 1 h (fast reduction) or 24 h (slow reduction). An extreme salinity reduction, directly from 36 to 18 psu, occurring within 1 h on 6 dph (drastic) was also tested. Results showed that larvae experiencing the slow or fast reduction were able to use their maternally supplied energy reserves more efficiently than larvae reared under full strength salinity, leading to improved growth and survival. At mouth opening (6 dph), control larvae showed upregulated *dio3* (deiodination) expression, confirming the salinity sensitivity of this mechanism, while at the first-feeding stage (12 dph), they showed increased *nkcc1a* expression, indicating upregulated transcellular ion transport. Irrespective of salinity, genes relating to water transport (*aqp1dup*) and skeletogenesis (*bmp2a*) showed highest expression at larval hatch (0 dph) and decreased with increasing age, revealing their potential importance during earlier development. Moreover, a core group of genes linked to the molecular mechanisms of neurogenesis (*neurod4*, *ngn1*) and heart development (*nppb*, *nppa*), peaked around the mouth opening stage (6 dph). The majority of genes, relating to almost all key molecular mechanisms targeted in this study, peaked at the first-feeding stage, corresponding to a timing of organogenesis refinement and/or specific functional tissue development, in order to ensure optimal transition from endogenous to exogenous feeding. Another core group of genes, which were not affected by larval age, were linked to the molecular mechanisms of energy metabolism (*atp6*, *cox1*), thyroid metabolism/deiodination (*dio1*, *dio3*), skeletogenesis (*bmp5*) and water transport (*aqp3*), revealing their basal importance throughout early eel ontogeny. To conclude, we demonstrate the applicability of an ecologically viable and economically efficient salinity reduction protocol for eel larvi-culture and describe a genetic (parental) programming of molecular mechanisms and intrinsic batch-specific sensitivity to environmental drivers that need to be considered in future eel aquaculture.

Key words: *Anguilla anguilla*; fish; aquaculture; environmental biology; molecular ontogeny; gene expression

Introduction

The European eel, *Anguilla anguilla*, is considered critically endangered, which is directly linked to declines in recruitment and affected by anthropogenic interference, climatic changes, and unsustainable aquaculture practice, which are capture-based and rely on glass eel fisheries (Pike et al 2020). As such, there is an urgent need to close the life cycle for this species in captivity and develop assisted reproduction techniques to support sustainable aquaculture development and conservation measures. The life cycle of the European eel includes oceanic and continental phases but is in its complexity still not fully understood. A widely held view is that spawning takes place in the Sargasso Sea area, which is supported by the presence of the youngest larvae ever caught (Schmidt, 1923, Scoth and Tesch, 1984; Castonguay and McCleave, 1987), but questions remain, as the earliest life stages (embryos and yolk-sac larvae) have still not been encountered in nature (Kuroki et al 2017).

In this regard, increased scientific inquiry has steadily advanced development of assisted reproduction methods for a stable production of viable gametes and offspring (Butts et al 2014; Müller et al 2016; Sørensen et al 2016; Benini et al 2018; Tomkiewicz et al 2019). However, as natural conditions cannot be accurately consulted, the lack of information regarding preferred environmental conditions during the earliest life stages (i.e. egg to first-feeding), challenge the development of hatchery techniques and technology. Targeted experimental research efforts, addressing these challenges to identify optimal rearing conditions, such as temperature (Okamura et al 2007; Ahn et al 2012; Politis et al 2017), light (Politis et al 2014), and salinity (Okamura et al 2009; Ahn et al 2015; Sørensen et al 2016, Politis et al 2018a), but also explore first-feeding options (Kagawa et al 2005; Okamura et al 2013; Butts et al 2016; Politis et al 2018b), have substantiated insights throughout ontogeny and led to significant progress towards closing the life cycle of anguillid eels.

Regarding salinity, as eel offspring naturally occur in the ocean (Castonguay and McCleave, 1987), they should be biologically equipped to develop in the hyper-osmotic seawater environment. Interestingly though, it was shown that reducing salinity towards iso-osmotic conditions during early life history in the hatchery, results in better growth and survival of Japanese (Okamura et al 2009) and European (Politis et al 2018a) eel larvae. This has so far been attributed to an energy surplus associated to lower resource allocation for osmoregulation, which can then be utilized more efficiently to ease development and survival (Okamura et al 2016; Politis et al 2018a). In this context, molecular mechanisms such as cell ion homeostasis (sodium potassium chloride ion co-transporters), water transport (aquaporins), cell repair in response to physiological stress (heat shock proteins), and energy metabolism (oxidative phosphorylation pathway) have previously been chosen as biomarkers to explore physiological processes of balancing ion homeostasis and osmotic equilibrium in relation to salinity changes (Ahn et al 2015; Politis et al 2018a).

The objective of this study was to test different salinity reduction scenarios during captive produced European eel larvi-culture, using recirculating aquaculture systems (RAS) in a real hatchery context, with the main goal to assess optimal conditions for rearing eel larvae. Here, four different rearing protocols were applied. Starting on 3 days post hatch (dph), a reduction of 4 practical salinity units (psu) per day was applied to reach the desired salinity (18 psu) either i) within 1 h (*fast*) or ii) drip-wise over 24 h (*slow*). In an additional treatment, the salinity reduction from 36 to 18 psu was applied on 6 dph, with the change occurring within 1 h (*drastic*). In the last rearing treatment, salinity was kept constant at 36 psu (*control*). To elucidate larval performance thresholds as well as decipher functionality and timing of key molecular processes related to salinity, the expression patterns of genes associated to water transport (*aqp1dup*,

aqp3, *aqpe*), ion regulation (*nkcc1a*, *nkcc2a*, *nkcc2b*), thyroid metabolism (*thaa*, *thab*, *thbb*, *dio1*, *dio2*, *dio3*), neurogenesis (*neurod4*, *ngn1*), growth (*gh*, *igf1*, *igf2b*), skeletogenesis (*bmp2a*, *bmp2b*, *bmp5*), heart development (*nppa*, *nppb*, *npr1*, *npr2*), energy metabolism (*atp6*, *cox1*), stress (*crfr1*, *crfr2*), stress/repair (*hsp70*, *hsp90*), and immune system ontogeny or immune response (*mhc2*, *il1b*, *tlr2*) were investigated.

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. Yolk-sac larvae of European eel were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222).

Broodstock management

Female broodstock were wild-caught from Saltbækvig, Denmark (55°44'51.1"N 11°08'28.3"E), while all males originated from a commercial eel farm (Royal Danish Fish, Hanstholm, 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 integrated into a closed recirculation system, under a continuous flow rate per tank of ~10-15 L min⁻¹, low intensity light (~20 lux) and 12 h light/12 h dark photoperiod. Acclimatization took place over 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 and embryonic incubation

Overall, gamete production and handling followed procedures previously described in [Butts et al \(2014\)](#), [Sørensen et al \(2016b\)](#), and [Benini et al \(2018\)](#). To induce vitellogenesis female eels received weekly injections of salmon pituitary extract (Argent Chemical Laboratories, Washington, USA) at 18.75 mg kg⁻¹ body weight ([Müller et al 2016](#); [Tomkiewicz et al 2019](#)). To stimulate follicular maturation and induce ovulation, female eels received an additional injection of 17 α ,20 β -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 strip-spawned. Males received weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich Chemie, Steinheim, Germany) at 150 IU/fish. Prior to fertilization, they were given an additional injection and milt was collected ~12 h thereafter. Milt samples were pipetted into an immobilizing medium ([Penaranda et al 2010](#)) at a concentration of 1:99 ([Ohta et al., 1996](#)) and used for fertilization within 4 h of collection ([Butts et al 2014](#)).

Eggs from each female were “crossed” with a pool of milt from several males to create the different family crosses ($n = 3$ families). Eggs from each female were stripped into dry plastic containers and gametes were swirled together. Artificial seawater (20°C), prepared by using reverse osmosis filtration (Vertex Puratek 100 gpd RO/DI, Vertex Technologies Inc., CA, USA) and salted to 36 psu (Auaforest Reef Salt, Brzesko, Poland), was added for a gamete contact time of 5 min (Butts et al 2014; Sørensen et al 2016). Eggs/embryos were then incubated for 2 h in 15 L containers filled with the above described artificial seawater before the buoyant eggs were transferred to 60 L black conical egg incubators, supplied with conditioned filtered seawater (Politis et al 2018b) at a flow through rate of $\sim 350 \text{ ml min}^{-1}$. Gentle aeration was added after ~ 10 hpf, while temperature was lowered to $18 \pm 0.5^\circ\text{C}$ for better embryonic development (Politis et al., 2017). Light was kept at a low intensity of ~ 10 lux (Politis et al., 2014) and sinking dead eggs were purged from the bottom valve of each incubator. At ~ 48 hpf, aeration was stopped and larvae hatched at ~ 56 hpf.

Experimental design and conditions

The experiment was repeated 3 times, each time using a different family cross – in total: $4 \text{ treatments} \times 3 \text{ family crosses} \times 3 \text{ replicates} = 36 \text{ experimental units}$. After hatch, larvae (~ 1000 individuals per replicate) were randomly distributed into replicated 8 L Acrylic Kreisel tanks ($n = 3$) for each family and each treatment. Eel larvae were reared throughout the endogenous feeding stage (from 0 to 12 dph). The Kreisel tanks of each treatment were connected to a separate RAS unit (Fig 1), where flow rates of conditioned filtered seawater were kept at $\sim 10 \text{ L min}^{-1}$. Each RAS unit facilitated a sump reservoir of $\sim 1 \text{ m}^3$, from where water entered a 1 m^3 biofilter with 750 m^2 substrate surface (RK elements, RK BioElements, Skive, Denmark), followed by a trickle filter 200 m^2 (BioBlock 200, Expo-Net Denmark, Hjørring, Denmark) and then re-entered the bottom reservoir. Here, a protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic GmbH, Bissendorf, Germany) was included for removal of waste protein. A UV lamp was also included to treat the newly filtered water (11W, JBL ProCristal, Neuhofen, Germany).

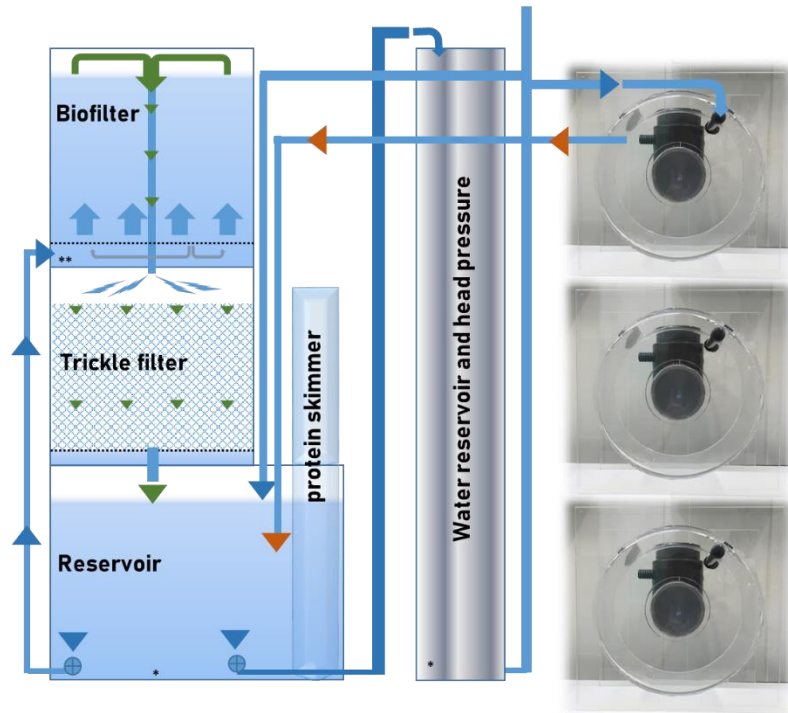


Fig 1 Eel larvae were reared throughout the endogenous feeding stage (from 0 to 12 days post hatch (dph)) in 8 L Acrylic Kreisel tanks ($n = 3$ for each family and each treatment). The Kreisel tanks of each treatment were connected to a separate Recirculating Aquaculture System (RAS) unit, where flow rates of conditioned filtered seawater were kept at $\sim 10 \text{ L min}^{-1}$. Each RAS unit facilitated a sump reservoir of $\sim 1 \text{ m}^3$, from where water entered a 1 m^3 biofilter with 750 m^2 substrate surface (RK elements, RK BioElements, Skive, Denmark), followed by a trickle filter 200 m^2 (BioBlock 200, Expo-Net Denmark, Hjørring, Denmark) and then re-entered the bottom reservoir. Here, a protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic GmbH, Bissendorf, Germany) was included for removal of waste protein. A UV lamp was also included to treat the newly filtered water (11 W, JBL ProCristal, Neuhofen, Germany).

Larvae were reared under four different salinity reduction protocols (Tab 1). In the **control** treatment, salinity was kept constant at 36 psu, which is closely resembling the salinity conditions encountered in the assumed spawning area in the Sargasso Sea. In the **fast** treatment, starting on day3, salinity was reduced in steps of 4 psu per day, by replacing 100 L of seawater with freshwater within 1 h. This treatment resembles one of the previously suggested reduction regimes for eel larvae (Politis et al 2018a). In the **slow** treatment, starting on day3, salinity was reduced in steps of 4 psu per day, by drip-wise replacing 100 L of seawater with freshwater within 24 h. We hypothesized that this treatment represents a more gradual and gentle salinity reduction. Lastly, in the **drastic** treatment, larvae were originally reared together with the control at 36 psu and moved on day6 to the 50% salinity reduced RAS of the fast treatment. Throughout the experiment, temperature was kept at $18 \pm 0.5^\circ\text{C}$ and pH at 8.1 ± 0.5 .

Table 1: European eel (*Anguilla anguilla*) larvae reared in 4 different salinity treatments. No reduction - constant at 36 psu (**control**) and in 3 further scenarios, where salinity reduction was applied at rates of 4 practical salinity units (psu) per day either within 1 h (**fast**) or 24 h (**slow**) initiated on 3 days post hatch. An extreme salinity reduction from 36 to 18 psu occurring within 1 h on 6 dph (**drastic**) was also tested.

| | | Salinity treatments in practical salinity units (psu) | | | |
|------------------------------|----|---|--------------------------------|---------------------------------|---------------------------------------|
| | | control no reduction | slow 4 psu/day within 1h | fast 4 psu/day within 24h | drastic 36 -> 18 psu within 1 h |
| Age in days post hatch (dph) | 0 | 36 | | | |
| | 1 | | 36 | 36 | 36 |
| | 2 | | | | |
| | 3 | | 32 | 32 | |
| | 4 | | 28 | 28 | |
| | 5 | | 24 | 24 | |
| | 6 | | 20 | 20 | |
| | 7 | | | | 18 |
| | 8 | | | | |
| | 9 | | | | |
| | 10 | | 18 | 18 | |
| | 11 | | | | |
| | 12 | | | | |

Data collection

Survival

Larval survival was monitored daily, where every day dead larvae were counted and removed from all experimental units. Additionally, all larvae at the end of the experiment as well as all sampled larvae from each experimental unit were enumerated and recorded. Larval cumulative mortality/survival was then calculated as a percentage from hatch until 12 dph.

Biometrics

Larval development (biometry) was followed from hatch and throughout the endogenous feeding stage (0, 6, and 12 dph), where ~10 larvae from each replicate (n = 3), family (n = 3), and treatment (n = 4) were randomly sampled and digitally imaged using a digital camera (Digital Sight DS-Fi2, Nikon Corporation, Japan) attached to a zoom stereomicroscope (SMZ1270i, Nikon Corporation, Japan). All endogenously feeding larvae were anesthetized using tricaine methanesulfonate (MS-222; Sigma-Aldrich, Missouri, USA) prior to digital imaging. NIS-Elements-D analysis software (Version 3.2) was used to analyze the larval images (Nikon Corporation, Japan), where total body and oil-drop area were measured for each larva. Larval growth and oil-drop utilization rate were measured from the change in body and oil-drop area, respectively. Growth efficiency was then measured by dividing the increase in body area by the corresponding decrease in oil-drop area (according to [Politis et al 2014](#)). Moreover, the severity of pericardial edema was calculated using the ratio between edema/neurocranium heights according to [Okamura et al 2009](#).

Gene expression

For molecular analysis, ~30 larvae from each replicate, family cross, and treatment were randomly sampled at hatch and throughout the endogenous feeding stage (0, 6, and 12 dph). Those larvae were recorded, euthanized using MS-222, preserved in RNAlater Stabilization Reagent and kept at -20°C following the procedures 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 and purity were determined by spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany) and then transcribed using the qScript™ cDNA Synthesis Kit (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipe out step [PerfeCta DNase I Kit (Quantabio, Germany)]. The expression levels of target genes were determined by quantitative real-time PCR (RT-qPCR), using specific primers (Table 2). 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 T_m of 60°C. Expression of genes in each larval sample from 2 randomly selected replicates, from each family cross (n=3), treatment, and larval age (0, 6, and 12 dph) were analysed in two technical replicates using the qPCR Biomark™ HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips). A pre-amplification step was performed with a 500 nM primer pool of all primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3µL 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 50µM. The chip was run according to the Fluidigm 96.96 PCR protocol with a T_m of 60°C. The relative quantity of target gene transcripts was normalized (ΔCT) to the geometric mean of the 2 most stable (housekeeping) genes. The *ef1a* and *npr3* genes were chosen as housekeeping genes, after qBase+ software revealed that these mRNA levels were stable throughout analyzed samples ($M < 0.4$); M gives the gene stability and $M < 0.5$ is typical for stably expressed reference genes (Hellemans et al 2007). Coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001).

Statistical analysis

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted values) to ensure they met model assumptions. Data were log(10) or arcsine square root (percentage data) transformed to meet these assumptions when necessary. Alpha was set at 0.05 for testing main effects and interactions. Treatment means were contrasted using Tukey's Honest Significant Difference test. Body area, oil droplet area, growth rate, oil droplet utilization, growth efficiency, edema severity and survival at 12 dph as well as gene expression (33 genes) at each age (6 and 12 dph) were analyzed using a series of mixed model factorial ANOVA models (PROC GLM). The main model variables were salinity treatment (fixed effect), family (random effect), and the salinity treatment × family interaction (random effect). Variance components (VC) for random effects were generated in PROC VARCOMP using the Restricted Maximum Likelihood (REML) estimation method and expressed as a

percentage. The mean and standard error for each salinity treatment and family effect were calculated using PROC MEANS. Additionally, a series of mixed effects models were run to investigate how gene expression changes over time and throughout early larval ontogeny for each salinity regime (control, slow, fast, drastic). These ANOVA models included the larval age (0, 6, 12 dph) fixed effect, the random family effect and the random age × family interaction.

Table 2. European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR and designed based on sequences available on Genbank databases. The table lists function, gene name and abbreviation as well as sequences for forward and reverse primers.

| Function | Gene | Abbreviation | 5' forward 3' | 5' reverse 3' |
|--------------------|--|----------------|-----------------------|------------------------|
| Housekeeping | Atrial natriuretic peptide receptor 3 | <i>npr3</i> | AACCTCCACGTGTAGACTG | TGACCAGAATTGCTCCCTCTT |
| | Elongation factor 1 | <i>ef1</i> | CTGAAGCCTGGTATGGTGGT | CATGGTGCATTCCACAGAC |
| stress - repair | Heat shock protein 70 | <i>hsp70</i> | TCAACCCAGATGAAGCAGTG | GCAGCAGATCCTGAACATTG |
| | Heat shock protein 90 | <i>hsp90</i> | ACCATTGCCAAGTCAGGAAC | ACTGCTCATCGTCATTGTGC |
| Growth | Insulin-like growth factor 1 | <i>igf 1</i> | TTCCTTTAGCTGGGCTTTG | AGCACCAGAGAGAGGGTGTG |
| | Insulin-like growth factor 2b | <i>igf 2b</i> | AAAGCTTTGGGACAGCTTCA | CGCAGCTGTGTACGTGAAAT |
| | Growth hormone | <i>gh</i> | TGAACAAGGGCATCAATGAA | CGGAGCTTTCCACATCCTC |
| Stress | Corticotropin-releasing factor receptor 1 | <i>crfr1</i> | GCATGAAGAGGATGAAGGCG | ATAGATGGGATCGGCACCTG |
| | Corticotropin-releasing factor receptor 2 | <i>crfr2</i> | CAGGAGGAGGAAGATGGCTG | CTGGAACCTGATCACCACCT |
| Skeletogenesis | Bone morphogenetic protein 2a | <i>bmp-2a</i> | TCTGTGAAGAGGACCAGCATG | CTGGATGGCAGACGAGGG |
| | Bone morphogenetic protein 2b | <i>bmp2b</i> | AGCAAGCTGGACGAGAAGAA | CGTATGATTGGCACTCGT |
| | Bone morphogenetic protein 5 | <i>bmp-5</i> | CGCAATAATCCAGTCTCTGCC | GCACAAGGGAGGAGACCAAA |
| energy | Cytochrome-C-Oxidase | <i>cox-1</i> | CTACTCCTCTCCCTGCCAGT | CTTCTGGGTGGCCGAAGAAT |
| | ATP synthase F0 sub-unit 6 | <i>atp6</i> | GGCCTGCTCCCATACACATT | GACTGGTGTTCCTTCTGGCA |
| Ion transport | Na ⁺ K ⁺ 2Cl ⁻ Cotransporter 1α | <i>nkcc1a</i> | CCAAGGCTCAGATCTTCTG | TTTCCGAATGGTAACCGAAG |
| | Na ⁺ K ⁺ 2Cl ⁻ Cotransporter 2α | <i>nkcc2a</i> | ACGTGGTTGGGTTTTAGAG | GTGAGATCCCCAAAAGCAAA |
| | Na ⁺ K ⁺ 2Cl ⁻ Cotransporter 2b | <i>nkcc2b</i> | AGCCAAAGTGGTGGATGTT | TGTCAGCCTCTCCAGTTCT |
| Heart development | Atrial natriuretic peptide A | <i>nppa</i> | CCTGAAGGCACACGACTACT | ACCACACCAGACGACCTTTT |
| | Atrial natriuretic peptide B | <i>nppb</i> | ACAGCGACAAAATGGACCAAC | TTCTTTGAGGTTGCTCGCT |
| | Atrial natriuretic peptide receptor 1 | <i>npr1</i> | ACCTCCATCAGCACAGGATC | GCATGTACACCTCCCTCAGT |
| | Atrial natriuretic peptide receptor 2 | <i>npr2</i> | AAACCCGATGCGTTCCTTGG | CGAGTGTAGGTAATGGGCGA |
| neurogenesis | Neuronal Differentiation 4 | <i>neurod4</i> | TTCCTGTCTCGCACCAGTA | AAGGAGTCGAAGGCCATGTC |
| | Neurogenin 1 | <i>ngn1</i> | CAGGATGCACAACCTCAATG | TGCAATTCGGATTGTCTCTG |
| thyroid metabolism | Thyroid Hormone Receptor αa | <i>thaa</i> | GCAGTTCAACCTGGACGACT | CCTGGCACTTCTCGATCTTC |
| | Thyroid Hormone Receptor αb | <i>thab</i> | GAAGCCTTCAGCGAGTTTAC | ACAGCCTTTCAGGAGGATGA |
| | Thyroid Hormone Receptor βb | <i>th8b</i> | GAAGACTGAGCCCTGAGGTG | AGGTAATGCAGCGGTAATGG |
| | Deiodinase 1 | <i>dio1</i> | AGCTTTGCCAGAACGACTGT | TTCCAGAACTTTCGCACCT |
| | Deiodinase 2 | <i>dio2</i> | GAAGAGGAGGATCGCCTACC | GCACTCTACCCTCCGTCAAA |
| | Deiodinase 3 | <i>dio3</i> | TACGGGGCGTATTTTGAGAG | GCTATAACCTCCGGACCTC |
| Immune response | Major histocompatibility complex, Class II | <i>mhc2</i> | TCAAATTGACCTGGCTGAGAG | TTTCCATTAGCCAGCTCCTC |
| | Interleukin 1β | <i>il18</i> | ATTGGCTGGACTTGTGTTC | CATGTGCATTAAGGCTGACCTG |
| | Toll like receptor 2 | <i>tlr2</i> | TGGTTCTGGCTGTAATGGTG | CGAAATGAAGGCATGGTAGG |
| aquaporins | Aquaporin 1 duplicate | <i>aqp1dup</i> | GAATTCCTGGCAACCTTTCA | CAAGATGACCCAGACCCACT |
| | Aquaporin 3 | <i>aqp3</i> | GCTCTCATGGCTTGTTCCTC | AAGGTCACAGTGGGGTTTCA |
| | Aquaporin e | <i>aqpe</i> | TGGGCAGCTGACAGTAACAG | AATCACCTGGTCCACAAAGC |

Results

Survival

Eel larval survival until 12 dph, was significantly ($p < 0.01$) improved when larvae experienced the slow ($55 \pm 8\%$) and fast ($51 \pm 7\%$) salinity reductions, compared to the control ($27 \pm 5\%$) and drastic ($24 \pm 3\%$) treatments (Fig 2). Here, a significant ($p < 0.01$) effect of family cross was observed, explaining 59.6% of the total variance. No statistically significant treatment \times family interaction was observed.

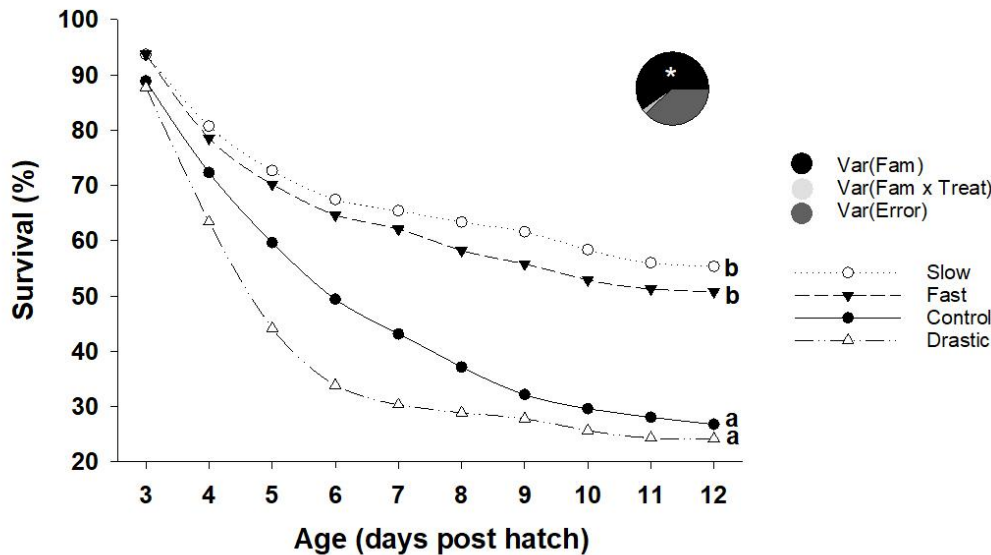


Figure 2: Survival (%) of European eel (*Anguilla anguilla*) larvae reared over 12 days post hatch (dph) under four different salinity scenarios: i) from 3 dph and onwards, reduction of 4 practical salinity units (psu) per day - change within 1 h (*fast*); ii) from 3 dph and onwards, reduction of 4 psu/day - change over 24 h drip-wise (*slow*); iii) on 6 dph, reduction from 36 to 18 psu - change within 1 h (*drastic*); and iv) no reduction - constant salinity at 36 psu (*control*). Values represent means of 3 family crosses. The main model variables were salinity treatment (fixed effect), family (random effect), and the salinity treatment \times family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components. Variance components were generated using the Restricted Maximum Likelihood estimation method and expressed as a percentage. Alpha was set to 0.05.

Biometrics

Larval body area on 12 dph was significantly ($p < 0.001$) larger when salinity was decreased slow (4.12 ± 0.1 mm²) and fast (4.06 ± 0.1 mm²), compared to the drastic (3.67 ± 0.1 mm²) and control (3.54 ± 0.1 mm²) treatments (Fig 3A). At the same stage, significantly ($p < 0.05$) more energy reserves (in terms of oil droplet area; Fig 3B) were available for larvae experiencing the slow reduction (0.015 ± 0.001 mm²) compared to the full-strength seawater (control; 0.012 ± 0.001 mm²). Eel larvae grew significantly ($p < 0.001$) more when salinity was decreased slow (0.185 ± 0.007 mm²/d) and fast (0.181 ± 0.006 mm²/d), compared to the drastic (0.148 ± 0.007 mm²/d) and control (0.137 ± 0.010 mm²/d) treatments (Fig 3C). At the same time, no statistically significant difference in energy (oil droplet) reserve utilization (0.007 ± 0.0001 mm²/d) was observed among treatments (Fig 3D), which resulted in significantly ($p < 0.001$) higher growth efficiency in eel larvae experiencing the slow and fast reduction, compared to the drastic and control treatments (Fig 3E). Moreover, the heart edema severity ratio (Fig 3F) was significantly ($p < 0.01$) increased for eel larvae reared in all salinity reduced treatments (slow, fast and drastic) compared to the full-strength seawater (control). Furthermore, the VC for family were significant for all biometrical parameters, explaining 67.3%

($p < 0.0001$), 21.1% ($p < 0.05$), 57.5% ($p < 0.001$), 77.6% ($p < 0.0001$), 66.5% ($p < 0.001$) and 69.1% ($p < 0.001$) of the total variance observed in body area, oil droplet area, growth, oil droplet utilization, growth efficiency and heart edema severity ratio, respectively. No statistically significant treatment \times family interactions were observed.

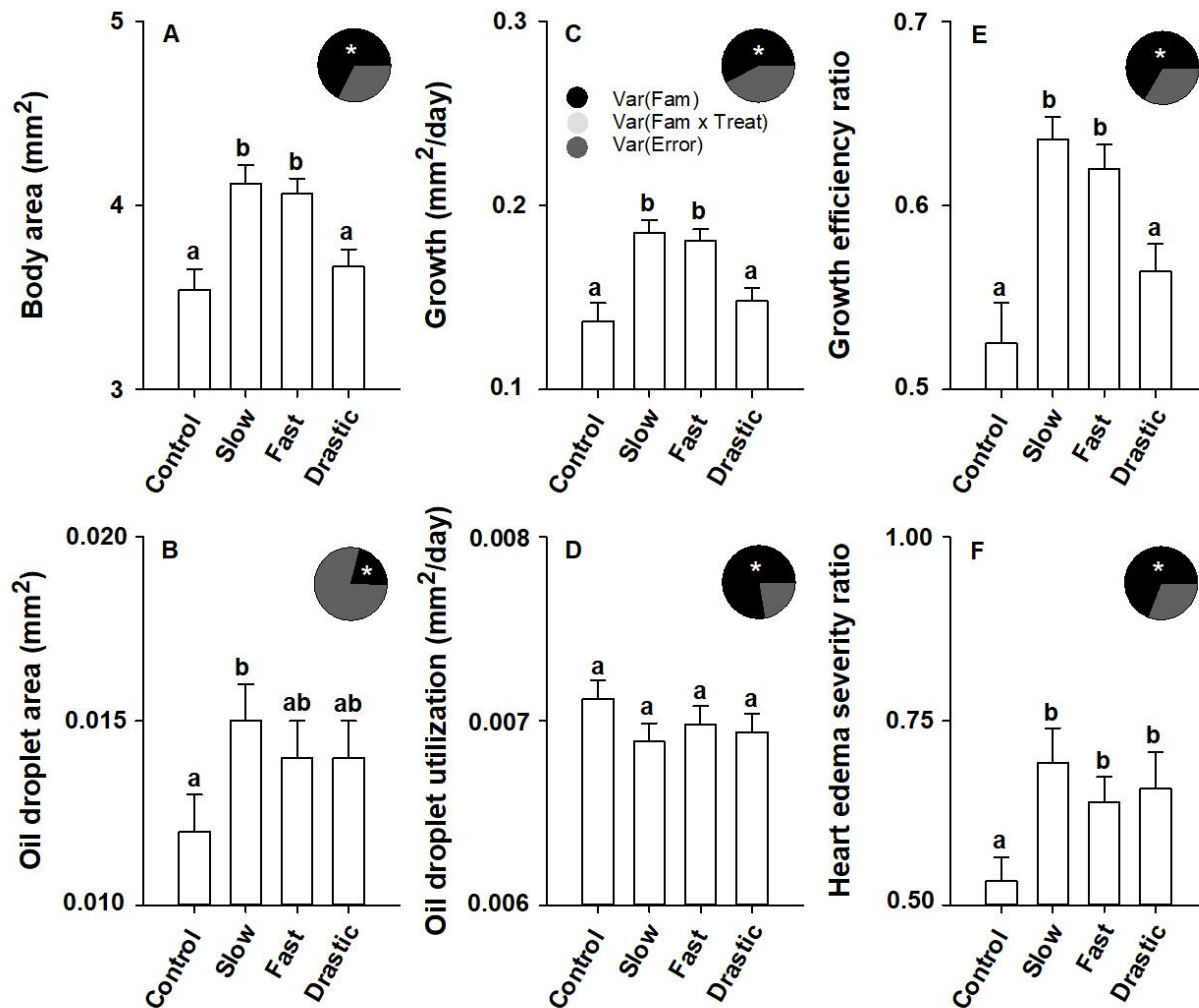


Figure 3: Body area (A), oil droplet area (B), growth/day (C), oil droplet utilization/day (D), growth efficiency (E) and heart edema severity ratio (F) of European eel (*Anguilla anguilla*) larvae reared over 12 days post hatch (dph) under four different salinity scenarios: i) from 3 dph and onwards, a reduction of 4 practical salinity units (psu) per day - change within 1 h (fast); ii) from 3 dph and onwards, reduction of 4 psu/day - change over 24 h drip-wise (slow); iii) on 6 dph, reduction from 36 to 18 psu - change within 1 h (drastic); and iv) no reduction - constant salinity at 36 psu (control). Values represent means of 3 family crosses. The main model variables were salinity treatment (fixed effect), family (random effect), and the salinity treatment \times family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components. Variance components were generated using the Restricted Maximum Likelihood estimation method and expressed as a percentage. Alpha was set to 0.05.

Gene expression

In all treatments, 2 of 33 genes (~6%) relating to water transport (*aqp1dup*) and skeletogenesis (*bmp2a*) showed highest expression at larval hatch (0 dph) and significantly ($p < 0.01$) decreased with increasing age (Fig 4). Moreover, on 6 dph (at mouth opening), 6 genes (~16%) significantly ($p < 0.05$) peaked in the slow

and fast treatments, while 7 genes (~18%) significantly ($p < 0.05$) peaked in the control and drastic treatments. Here, a core group of genes, showing the same expression pattern and peaking at this stage in all treatments, were linked to the molecular mechanisms of neurogenesis (*neurod4*, *ngn1*) and heart development (*nppb*, *nppa*). The other genes, significantly but irregularly peaking among treatments on 6 dph, were relating to skeletogenesis (*bmp2b*), thyroid metabolism (*thab*, *thbb*), heart development (*npr2*) and immune system ontogeny or immune response (*il1b*, *tlr2*). Furthermore, the majority of genes, relating to almost all targeted key molecular mechanisms (except neurogenesis and skeletogenesis), peaked on 12 dph. Here, 16 genes (~48%) in the control, 13 (~39%) in the slow, 17 (~51%) in the fast and 14 (~42%) in the drastic treatments, significantly ($p < 0.05$) peaked at this point, the first-feeding stage. On the other hand, the expression patterns of 8 genes (~24%) in the control, 12 genes (~36%) in the slow, 8 genes (~24%) in the fast and 10 genes (~30%) in the drastic treatments were not significantly affected by larval age (Tab 3). This revealed another core group of genes, showing a constant expression pattern over time in all treatments, which were linked to the molecular mechanisms of energy metabolism (*atp6*, *cox1*), deiodination (*dio1*, *dio3*), skeletogenesis (*bmp5*) and water transport (*aqp3*). The other genes, showing no significantly different expression pattern over time, were relating to skeletogenesis (*bmp2b*), deiodination (*dio2*), water transport (*aqpe*), ion transport (*nkcc1a*), stress response (*crfr2*) and immune system ontogeny or immune response (*il1b*, *mhc2*), with no regular pattern among treatments.

At mouth opening (6 dph), larvae reared in full strength seawater (control) showed significantly ($p < 0.05$) increased expression of *dio3* (deiodination), while at the first-feeding stage (12 dph), they showed significantly ($p < 0.05$) increased expression of *nkcc1a* (ion transport) compared to the salinity reduced treatments (Table 4). Moreover, the VC analysis revealed differing reaction norms relative to the environmental variable (salinity) investigated. As presented in Table 4, on 6 dph (mouth opening), the expression patterns of 13 genes (~39%), associated to immune response (*il1b*, *mhc2*, *tlr2*), deiodination (*dio1*, *dio2*, *dio3*), skeletogenesis (*bmp2a*), stress (*crfr1*, *crfr2*), growth (*gh*, *igf1*), ion regulation (*nkcc2a*) and heart development (*npr1*) were significantly ($p < 0.05$) influenced by “family”. At the same stage, 12 genes (~36%) associated to stress/repair (*hsp90*), immune response (*mhc2*), neurogenesis (*neurod4*), deiodination (*dio2*), thyroid metabolism (*thaa*, *thab*, *thbb*), energy metabolism (*atp6*), skeletogenesis (*bmp2b*, *bmp5*), growth (*igf2b*) and ion regulation (*nkcc2b*) were significantly ($p < 0.05$) influenced by the “family × treatment” interaction. Similarly, on 12 dph (first-feeding), the expression patterns of 14 genes (~42%) associated to water transport (*aqp3*, *aqpe*, *aqp1dup*), immune response (*il1b*, *mhc2*, *tlr2*), neurogenesis (*neurod4*), deiodination (*dio1*, *dio2*, *dio3*), ion regulation (*nkcc1a*, *nkcc2a*, *nkcc2b*) and heart development (*nppa*) were significantly ($p < 0.05$) influenced by “family”. At the same stage, 5 genes (~15%) associated to water transport (*aqp3*), immune response (*il1b*), thyroid metabolism (*thbb*), skeletogenesis (*bmp5*) and heart development (*nppb*) were significantly ($p < 0.05$) influenced by the “family × treatment” interaction.

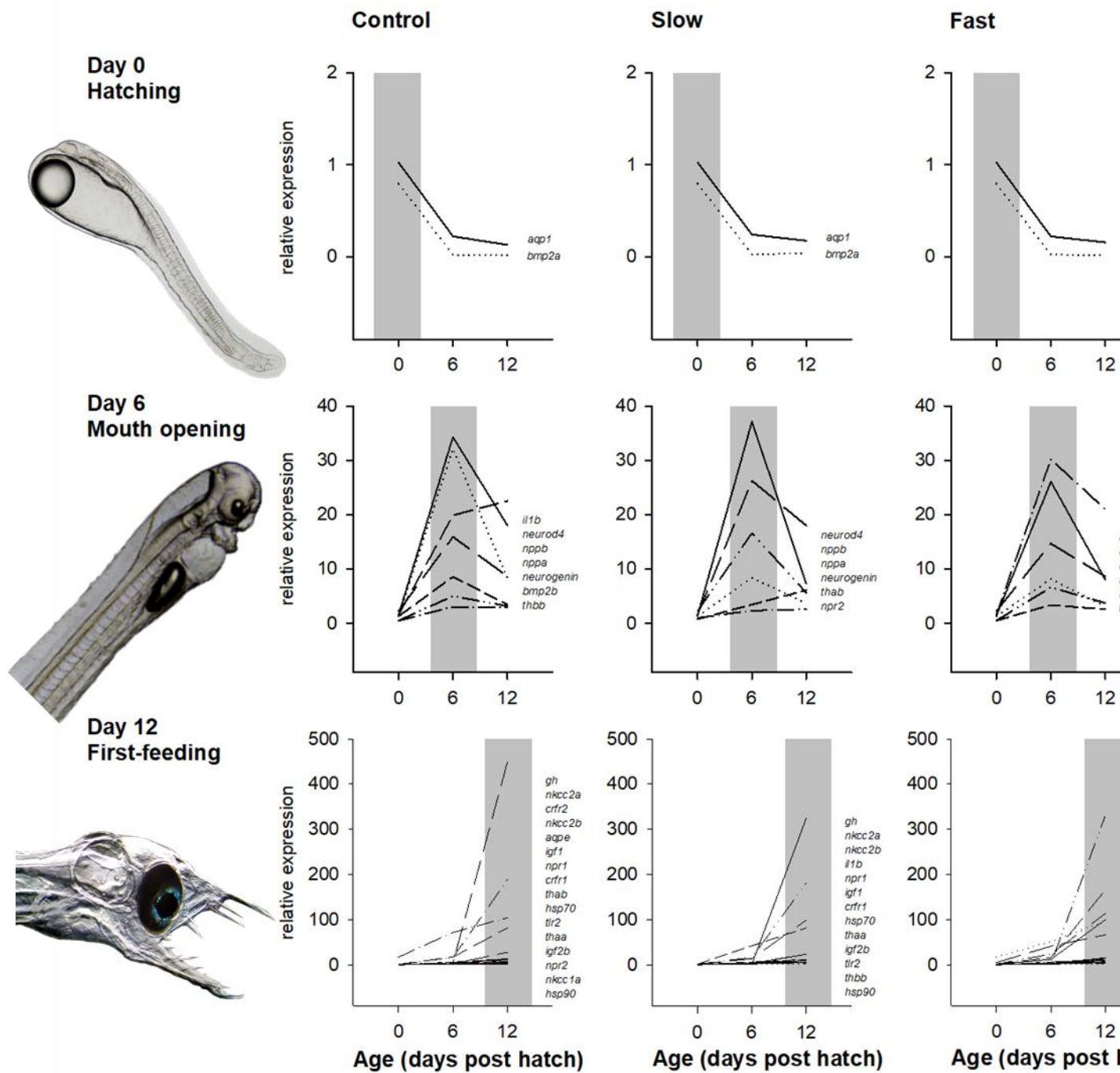


Figure 4: Expression patterns of genes linked to key molecular mechanisms (ion regulation, water transport, thyroid metabolism, deiodination, heart development, stress, stress/repair, immune response, growth, skeletogenesis, energy metabolism, and neurogenesis) throughout early larval ontogeny of European eel, *Anguilla anguilla*. Larvae were reared over 12 days post hatch (dph) under four different salinity scenarios: i) from 3 dph and onwards, a reduction of 4 practical salinity units (psu) per day - change within 1 h (fast); ii) from 3 dph and onwards, reduction of 4 psu/day - change over 24 h drip-wise (slow); iii) on 6 dph, reduction from 36 to 18 psu - change within 1 h (drastic); and iv) no reduction - constant salinity at 36 psu (control). Values represent means of 3 family crosses. Genes were grouped into 3 expression patterns, either significantly peaking at hatch (0 dph), at mouth opening (6 dph) or first-feeding (12 dph), elucidating timing and functionality of the associated mechanisms. Alpha was set to 0.05.

Table 3: Overview of statistical analysis relating to the effect of European eel, *Anguilla anguilla* larval age on expression patterns of genes relating to different key molecular mechanisms at each treatment. Larvae were reared over 12 days post hatch (dph) under four different salinity scenarios: i) from 3 dph and onwards, a reduction of 4 practical salinity units (psu) per day - change within 1 h (fast); ii) from 3 dph and onwards, reduction of 4 psu/day - change over 24 h drip-wise (slow); iii) on 6 dph, reduction from 36 to 18 psu - change within 1 h (drastic); and iv) no reduction - constant salinity at 36 psu (control). Genes were grouped into 3 expression patterns, either significantly peaking on 0 dph (↘), 6 dph (↑), and 12 dph (↗), or showing no significant pattern changes over time (↔) to elucidate timing and functionality of the associated mechanisms. Small letters represent significant differences among treatments. Alpha was set to 0.05.

| Function | Gene ID | Control | | | | Slow | | | | Fast | | | | Drastic | | | | | | | |
|--------------------|-------------------|---------|----|----|----|------|---------|---|----|------|---|--------|----|---------|----|---|--------|----|----|----|---|
| | | Age | 0 | 6 | 12 | Age | 0 | 6 | 12 | Age | 0 | 6 | 12 | Age | 0 | 6 | 12 | | | | |
| Stress/Repair | <i>hsp70</i> | 0.0001 | a | b | c | ↗ | 0.05 | a | ab | b | ↗ | 0.0131 | a | a | b | ↗ | 0.0033 | a | a | b | ↗ |
| | <i>hsp90</i> | 0.0182 | ab | a | b | ↗ | 0.0027 | a | a | b | ↗ | 0.0013 | b | a | c | ↗ | 0.0153 | ab | a | b | ↗ |
| Water transport | <i>aqp3</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ |
| | <i>aqpe</i> | 0.0426 | a | ab | b | ↗ | ns | . | . | . | ↔ | 0.0061 | a | a | b | ↗ | 0.0008 | a | a | b | ↗ |
| | <i>aqp1</i> | 0.0022 | b | a | a | ↘ | 0.0011 | b | a | a | ↘ | 0.0013 | b | a | a | ↘ | 0.0029 | b | a | a | ↘ |
| Immune response | <i>il1b</i> | 0.0267 | a | b | ab | ↑ | 0.0205 | a | ab | b | ↗ | 0.0445 | a | ab | b | ↗ | ns | . | . | . | ↔ |
| | <i>mhc2</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | 0.0357 | a | ab | b | ↗ | ns | . | . | . | ↔ |
| | <i>tlr2</i> | 0.0008 | a | b | c | ↗ | 0.0013 | a | b | c | ↗ | 0.0029 | a | b | c | ↗ | 0.0042 | a | b | b | ↗ |
| Neurogenesis | <i>neurod4</i> | 0.0107 | a | b | a | ↑ | 0.0036 | a | b | a | ↑ | 0.0009 | a | c | b | ↑ | 0.0043 | a | b | a | ↑ |
| | <i>neurogenin</i> | 0.0005 | a | c | b | ↑ | 0.0012 | a | c | b | ↑ | 0.0014 | a | b | a | ↑ | 0.0065 | a | b | a | ↑ |
| Deiodination | <i>dio1</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ |
| | <i>dio2</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | 0.0404 | a | ab | b | ↗ |
| | <i>dio3</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ |
| Thyroid metabolism | <i>thab</i> | 0.0002 | a | b | c | ↗ | 0.0044 | a | b | b | ↑ | 0.0005 | a | b | c | ↗ | 0.0030 | a | b | c | ↗ |
| | <i>thaa</i> | 0.0029 | a | a | b | ↗ | 0.05 | a | ab | b | ↗ | 0.0158 | a | ab | b | ↗ | 0.0046 | a | a | b | ↗ |
| | <i>thbb</i> | 0.0136 | a | b | b | ↑ | 0.0257 | a | ab | b | ↗ | 0.0031 | a | b | b | ↑ | 0.0022 | a | b | b | ↑ |
| Energy | <i>atp6</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ |
| | <i>cox1</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ |
| Skeletogenesis | <i>bmp2a</i> | 0.0002 | b | a | a | ↘ | <0.0001 | b | a | a | ↘ | 0.0002 | b | a | a | ↘ | 0.0002 | b | a | a | ↘ |
| | <i>bmp2b</i> | 0.0266 | a | b | ab | ↑ | ns | . | . | . | ↔ | 0.0342 | a | b | ab | ↑ | ns | . | . | . | ↔ |
| | <i>bmp5</i> | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ |
| Stress | <i>crfr1</i> | 0.0009 | a | b | c | ↗ | 0.0018 | a | b | c | ↗ | 0.0050 | a | a | b | ↗ | 0.0021 | a | b | c | ↗ |
| | <i>crfr2</i> | 0.0444 | a | ab | b | ↗ | ns | . | . | . | ↔ | 0.0032 | a | b | b | ↗ | 0.0372 | a | ab | b | ↗ |
| Growth | <i>gh</i> | 0.0060 | a | a | b | ↗ | 0.0029 | a | a | b | ↗ | 0.0015 | a | a | b | ↗ | 0.0087 | a | a | b | ↗ |
| | <i>igf1</i> | 0.0016 | a | a | b | ↗ | 0.0004 | a | b | c | ↗ | 0.0043 | a | a | b | ↗ | 0.0003 | a | b | c | ↗ |
| | <i>igf2b</i> | <0.0001 | a | b | c | ↗ | 0.0009 | a | b | c | ↗ | 0.0030 | a | a | b | ↗ | 0.0051 | a | a | b | ↗ |
| Ion regulation | <i>nkcc1a</i> | 0.0119 | a | a | b | ↗ | ns | . | . | . | ↔ | ns | . | . | . | ↔ | ns | . | . | . | ↔ |
| | <i>nkcc2a</i> | 0.0005 | a | b | c | ↗ | 0.0005 | a | b | c | ↗ | 0.0012 | a | a | b | ↗ | 0.0005 | a | b | c | ↗ |
| | <i>nkcc2b</i> | 0.0008 | a | b | c | ↗ | 0.0001 | a | b | c | ↗ | 0.0004 | a | b | c | ↗ | 0.0007 | a | b | c | ↗ |
| Heart development | <i>nppa</i> | 0.0007 | a | c | b | ↑ | 0.0008 | a | b | a | ↑ | 0.0106 | a | b | ab | ↑ | 0.0289 | a | b | ab | ↑ |
| | <i>nppb</i> | 0.0202 | a | b | b | ↑ | 0.05 | a | b | ab | ↑ | 0.0381 | a | b | ab | ↑ | 0.0262 | a | b | ab | ↑ |
| | <i>npr1</i> | 0.0004 | a | a | b | ↗ | 0.0426 | a | ab | b | ↗ | 0.0065 | a | a | b | ↗ | 0.0011 | a | a | b | ↗ |
| | <i>npr2</i> | 0.0005 | a | b | c | ↗ | 0.0046 | a | b | b | ↑ | 0.0335 | a | ab | b | ↗ | 0.0250 | a | b | b | ↑ |

Table 4: Variance component analysis on the expression patterns of genes linked to key molecular mechanisms of European eel, *Anguilla anguilla* larvae at the mouth opening stage on 6 days post hatch (dph) and the first feeding stage on 12 dph. Larvae were reared under four different salinity scenarios: i) from 3 dph and onwards, a reduction of 4 practical salinity units (psu) per day - change within 1 h (fast); ii) from 3 dph and onwards, reduction of 4 psu/day - change over 24 h drip-wise (slow); iii) on 6 dph, reduction from 36 to 18 psu - change within 1 h (drastic); and iv) no reduction - constant salinity at 36 psu (control). Values represent means of 3 family crosses. The main model variables were salinity treatment (fixed effect), family (random effect), and the salinity treatment × family interaction (random effect). Alpha was set to 0.05.

| Function | Gene ID | Mouth opening (day6) | | | First feeding (day12) | | |
|--------------------|-------------------|----------------------|---------|-------------|-----------------------|--------|-------------|
| | | Treat | Fam | Fam x Treat | Treat | Fam | Fam x Treat |
| Stress/Repair | <i>hsp70</i> | ns | ns | ns | ns | ns | ns |
| | <i>hsp90</i> | ns | ns | <0.05 | ns | ns | ns |
| Water transport | <i>aqp3</i> | ns | ns | ns | ns | <0.05 | <0.001 |
| | <i>aqpe</i> | ns | ns | ns | ns | <0.05 | ns |
| | <i>aqp1</i> | ns | ns | ns | ns | <0.01 | ns |
| Immune response | <i>il1b</i> | ns | <0.001 | ns | ns | <0.05 | <0.05 |
| | <i>mhc2</i> | ns | <0.0001 | <0.05 | ns | <0.05 | ns |
| | <i>tlr2</i> | ns | <0.001 | ns | ns | <0.05 | ns |
| Neurogenesis | <i>neurod4</i> | ns | ns | <0.01 | ns | <0.05 | ns |
| | <i>neurogenin</i> | ns | ns | ns | ns | ns | ns |
| Deiodination | <i>dio1</i> | ns | <0.001 | ns | ns | <0.001 | ns |
| | <i>dio2</i> | ns | <0.01 | <0.05 | ns | <0.001 | ns |
| | <i>dio3</i> | <0.05 | <0.01 | ns | ns | <0.01 | ns |
| Thyroid metabolism | <i>thab</i> | ns | ns | <0.0001 | ns | ns | ns |
| | <i>thaa</i> | ns | ns | <0.001 | ns | ns | ns |
| | <i>thbb</i> | ns | ns | <0.01 | ns | ns | <0.05 |
| Energy | <i>atp6</i> | ns | ns | <0.05 | ns | ns | ns |
| | <i>cox1</i> | ns | ns | ns | ns | ns | ns |
| Skeletogenesis | <i>bmp2a</i> | ns | <0.01 | ns | ns | ns | ns |
| | <i>bmp2b</i> | ns | ns | <0.05 | ns | ns | ns |
| | <i>bmp5</i> | ns | ns | <0.05 | ns | ns | <0.01 |
| Stress | <i>cfr1</i> | ns | <0.05 | ns | ns | ns | ns |
| | <i>cfr2</i> | ns | <0.05 | ns | ns | ns | ns |
| Growth | <i>gh</i> | ns | <0.01 | ns | ns | ns | ns |
| | <i>igf1</i> | ns | <0.05 | ns | ns | ns | ns |
| | <i>igf2b</i> | ns | ns | <0.01 | ns | ns | ns |
| Ion regulation | <i>nkcc1a</i> | ns | ns | ns | <0.05 | <0.01 | ns |
| | <i>nkcc2a</i> | ns | <0.05 | ns | ns | <0.05 | ns |
| | <i>nkcc2b</i> | ns | ns | <0.05 | ns | <0.01 | ns |
| Heart development | <i>nppa</i> | ns | ns | ns | ns | <0.05 | ns |
| | <i>nppb</i> | ns | ns | ns | ns | ns | <0.05 |
| | <i>npr1</i> | ns | <0.05 | ns | ns | ns | ns |
| | <i>npr2</i> | ns | ns | ns | ns | ns | ns |

Discussion

European eels are euryhaline species that undertake a catadromous reproductive migration resulting in eel offspring naturally occurring in a hypo-osmotic environment in the ocean. Here, plasma osmolality is lower than the environment (Lee et al 2013) and eel offspring need to maintain osmotic balance through desalting processes to counteract osmotic water loss. In this regard, eel larvae are expected to be genetically pre-programmed and equipped with molecular mechanisms to thrive in oceanic conditions. However, larvae produced via assisted reproduction and reared in the hatchery, benefit from an unnatural salinity reduction towards iso-osmotic conditions (Okamura et al 2009; Ahn et al 2015; Politis et al 2018a). As such, this study tested different salinity reduction scenarios during captive produced European eel larvi-culture, using RAS in a real hatchery context, with the main goal to assess optimal conditions for rearing eel larvae, but also explore the functionality and timing of key molecular mechanisms related to environmental parameters (such as salinity) and affecting eel larval performance.

Survival and biometrics

The results of the present study, show that eel larvae experiencing a salinity reduction are able to use their energy reserves, supplied by the mother (in terms of an oil droplet), more efficiently than larvae reared under full strength salinity, resembling the natural oceanic conditions. Consequently, the salinity reduction led to an increased growth rate and resulted in larger larvae, especially when salinity was reduced on 3 dph, irrespective of the change occurring slow (drip-wise over 24h) or fast (within ~1h). The same pattern was also observed in larval survival, where larvae experiencing the slow and fast salinity reduction, showed ~50% lower mortality. Similar survival rates have been observed in Japanese eel, where improved growth and survival were reported when larvae were reared in ~50% reduced salinity (Okamura et al 2009). Reducing seawater salinity (with an osmolality of ~1050 mOsm kg⁻¹ H₂O) to ~50%, facilitates an iso-osmotic environment for eel larvae, which were shown to have a tissue osmolality of 360 - 540 mOsm kg⁻¹ H₂O (Lee et al 2013). This, probably increases energy availability due to lower osmoregulatory and metabolic expenses, enabling the survival of weaker larvae, which would not survive in a high salinity environment (Okamura et al 2016). Interestingly though, in the current study, the survival of larvae experiencing a salinity reduction on 6 dph (drastic), followed the same pattern as the larvae experiencing no salinity reduction (control), which indicates that the process of “saving” the larvae should occur earlier. Moreover, it has been reported that rearing eel larvae at reduced salinity, causes deformities, such as pericardial edema in Japanese (Okamoto et al 2009) and European eel (Politis et al 2018a). The results of the present study are in agreement with those findings, as larvae with an increased pericardial edema severity ratio were observed in the salinity reduced treatments, irrespective of whether the reduction occurred slow, fast or drastic. Thus, we demonstrate and confirm that reducing salinity is a tradeoff process, which improves survival and growth but also induces an edematous state of the larval heart. In this regard, by following the expression patterns of genes relating to cardiovascular development, functionality, and homeostasis we aimed to fill some gaps in knowledge by providing the first documentation of ontogenetic timing and functionality of the heart development associated underlying molecular mechanisms in European eel larvae.

Heart development

Generally, teleost fishes have evolutionary experienced an additional whole genome duplication, which endowed them with extra adaptability to inhabit various aquatic habitats by developing key endocrine mechanisms, such as the natriuretic peptide system, which can be traced back to ancestral teleosts (such as the eel), representing a group of actors playing important roles in cardiovascular and osmoregulatory systems in vertebrates (Inoue et al 2005). Here, a number of natriuretic peptide receptors mediate the physiological response of different natriuretic peptide hormones in response to Na⁺ concentration (but not water, as known from mammals), to control adaptation to either seawater or freshwater (Takei et al 2014). The natriuretic peptide system can be active as early as embryogenesis, as the expression of genes encoding main actors of this mechanism have been confirmed during embryonic development of zebrafish, *Danio rerio* (Gong et al 2018). In the current study, genes encoding two natriuretic peptides (*nppa*, *nppb*) and two natriuretic peptide receptors (*npr1-2*) were expressed in recently hatched European eel larvae, revealing the involvement of this mechanism during early ontogeny. Interestingly, the expression of *nppa* and *nppb* peaked at 6 dph, demonstrating that key processes of cardiovascular development occur at this larval stage. The same pattern was observed in all salinity treatments, revealing either that the cardiovascular development occurring at this stage is part of a genetically pre-programmed primary organogenesis and not affected by environmental drivers, or that the mechanism and its sensitivity to extrinsic factors are not entirely matured.

Neurogenesis

The neurogenesis related factors (*neurod4* and *ngn1*) investigated in this study, belong to the helix-loop-helix family of transcriptional regulatory proteins (Morrison 2001). They work together to influence neuronal differentiation, but are also known to play a vital role in a wide range of developmental processes (Massari and Murre 2000). In mammals, *ngn1* binds to an enhancer regulatory element to form the so called E-box (enhancer-box) that acts as a protein-binding site for the transcription factor *neurod* to initiate transcription of further genes involved in neuronal differentiation (Sun et al., 2001). Sequences encoding for neurogenesis related genes, such as *ngn1* and/or *neurod4*, have also been identified in teleost species including zebrafish, *Danio rerio* (Hitchcock and Ochocinska 2003; Nakada et al., 2004) and brook lamprey, *Lampetra planeri* (Lara-Ramírez et al., 2015). The current study on European eel larvae, demonstrates that neuronal differentiation related gene expression of *ngn1* and *neurod4* was unaffected by salinity, but increased until reaching a peak on 6 dph and slowly decreased to basal levels again beyond that. Thus, we speculate that neuronal differentiation in European eel larvae (reared at 18°C) largely occurs at this stage, while non-neuronal cells (neuroglia) that maintain homeostasis and provide support and protection for neurons, are formed before and/or after this period. This would be largely in accordance with the previously described mechanism, where high levels of *ngn1* promote neurogenesis, whereas low levels of *ngn1* promote gliogenesis (Sun et al., 2001; Morrison 2001).

Growth and skeletogenesis

Processes such as growth, metabolism and development are regulated by the somatotrophic axis, a mechanism interlinking growth hormones (GH) and the closely connected (and regulated by GH), insulin-

like growth factors (IGF) (Reinecke et al 2005). This mechanism is involved in most physiological processes and has been documented in early life history stages of European eel (Rozenfeld et al 2016; Politis et al 2017), other eel species such as the ricefield eel (*Monopterus albus*) (Chen et al 2015) and the Japanese eel (Ozaki et al 2006), but also other fish species such as Senegalese sole (*Solea senegalensis*), zebrafish (*Danio rerio*), sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and rabbitfish (*Siganus guttatus*) (Perrot et al 1999; Ayson et al 2002; Campos et al 2013; Besseau et al 2013), suggesting that GHs and IGFs play a fundamental role throughout early development and ontogeny in teleosts. In the current study, we observed expression of *gh*, *igf1* and *igf2b* already at hatch, with an increasing pattern towards the first-feeding stage, further supporting the involvement of this key mechanism throughout European eel early ontogeny. Also of key importance during fish early ontogeny is the mechanism of skeletogenesis. Here, bone morphogenetic proteins (BMPs) are active osteogenesis actors, driving early skeletal development by inducing intermuscular bone and cartilage formation, but also promoting cell proliferation and differentiation during bone repair (Yang et al 2019). Interestingly, *bmps* can act as pleiotropic growth factors during development and as bone inducers during osteogenesis Marques et al 2016, but are also known to play multiple roles in tooth development in teleost fishes such as the zebrafish (*Danio rerio*), Mexican tetra (*Astyanax mexicanus*), and Japanese medaka (*Oryzias latipes*) (Wise and Stock 2006). In our study, the expression of *bmp2b* peaked at the larval mouth opening stage (in the control and slow salinity treatments), potentially revealing the involvement in European eel larval jaw/teeth formation. In contrast, expression of *bmp2a* decreased throughout ontogeny and reached basal levels during the mouth-opening and first-feeding stage, suggesting a minor role of this gene during this developmental period. In particular, bone morphogenetic protein 2 (*bmp2*) has been linked to osteogenesis and skeleton development, but may also contribute to different developmental and physiological processes in Senegalese sole (Marques et al 2014), while it is a secreted signaling molecule that acts as an inducer of bone formation and a regulator of embryonic development in *Sparus aurata* (Rafael et al 2006). As such, the spatiotemporal involvement of *bmp2* in European eel and potential stage specificities remain to be clarified. On the other hand, bone morphogenetic protein 5 (*bmp5*) is considered a promoter for intermuscular bones in blunt snout bream, *Megalobrama amblycephala* (Zhang et al 2018), but it is also known to be involved in the development of the sternum, thyroid cartilage, or the cells forming part of the vertebrae in mice (Ducy and Karsenty, 2000). In the current study, *bmp5* was steadily expressed in all larval stages, revealing a potential involvement in the ongoing processes of early ontogeny in European eel. Moreover, the VC analysis showed that the expression patterns of *gh*, *igf1* and *bmp2a* (at mouth opening) were driven by the “family” effect. In this regard, a “good genes” hypothesis, could translate into genetically preprogrammed “better” or more sensitive molecular mechanisms, endowed by the parents, to control those early ontogenetic processes of growth, skeletogenesis and development. On the other hand, the expression patterns of *igf2b*, *bmp2b*, and *bmp5* were driven by the genome × environment interaction, revealing that offspring originating from different parents will be equipped with different mechanism sensitivities to differentially respond to environmental stressors (such as salinity) and thus some eel larvae might grow and develop better/worse than others in response to salinity changes.

Thyroid metabolism

Early life history of fish has often been correlated to thyroid metabolism, which is under the control of the hypothalamus-pituitary-thyroid axis, where the thyroid gland is synthesizing thyroid hormone (Jarque and Piña, 2014). Thyroid hormone is synthesized as a precursor with weak bioactivity and can be converted into active hormones or inactive metabolites by three transmembrane proteins, the iodothyronine deiodinases (DIO1-3). 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). Deiodination has been linked to several functional and vital processes in several fish species, 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). Specifically, in eel, 2 subtypes of *thr* (*thra*, *thrβ*) and 3 subtypes of deiodinases (*dio1-3*) were previously identified in Japanese (Kawakami et al 2013) and European eels (Politis et al 2018c). In the present study, the expression patterns of several thyroid hormone pathway related actors (*thaa*, *thab*, *thbb*, *dio1*, *dio2*, *dio3*) were investigated and *dio3* was significantly influenced by salinity. This is precisely in accordance with previous observations, where the outer ring deiodination showed sensitivity to salinity in European eel (Politis et al 2018a) but also other fish species such as rainbow trout, *Oncorhynchus mykiss* (Orozco et al., 2002). Thus, our results further support the involvement of the thyroid endocrine system and the deiodination mechanism during early ontogeny of this species as well as its sensitivity to environmental parameters such as salinity.

Ion regulation and energy metabolism

In eel, osmoregulatory organs such as gills or kidneys are not fully or not at all developed during early life history (Sørensen et al 2016). In Japanese eel, it was discovered that larvae can drink as early as the day of hatch, while the role and timing of the intestine and rectum in controlling ion balance was confirmed by expression of osmoregulatory related genes, such as sodium potassium chloride ion ($\text{Na}^+\text{K}^+\text{Cl}^-$) cotransporters (Ahn et al 2015). The $\text{Na}^+\text{K}^+\text{Cl}^-$ cotransporters, which mediate the electroneutral cotransport of Na^+ , K^+ and Cl^- , are known to be involved in ion absorption and/or secretion as well as in cell volume homeostasis (Russell, 2000). Here, secretory (*nkcc1*) and absorptive (*nkcc2*) subtypes have previously been identified in adult and larval European eel (Cutler and Cramb 2001, Politis et al 2018a). Generally, the *nkcc1α* isoform is known for a wide range tissue distribution, whereas *nkcc1β* is predominantly expressed in the brain (Cutler and Cramb 2002). On the other hand, the *nkcc2α* isoform is rather restricted to renal tissues, whereas the *nkcc2β* isoform predominates in the intestine and urinary bladder (Cutler and Cramb 2008). In accordance to previous findings, in the current study, *nkcc2a* and *nkcc2b* were both significantly upregulated towards the first-feeding stage and peaked on 12 dph, confirming that the ion-regulatory ability increases throughout early ontogeny, which is probably linked to the increasing functionality of the associated tissue (kidney and gut, respectively). On the other hand, expression of *nkcc1a* was stable throughout larval ontogeny in the salinity reduced treatments but was significantly increased when larvae were reared in full strength seawater (control), indicating an upregulation of the active Na^+ , K^+ , and Cl^- transport. As this mechanism requires energy, an increased transcellular ion transport translates into increased cellular energy consumption when eel larvae are reared in 36 psu. In this regard, the expression levels of ATP-synthase and cytochrome-c-oxidase, involved in the oxidative phosphorylation (OXPHOS) pathway and generally associated to energy metabolism in teleost fishes (Bermejo-Nogales et al 2015), were constant throughout the entire developmental period, suggesting that energy production was stable and independent of salinity treatments. As such, considering

the increased ion regulation and osmotic demands (i.e. higher *nkcc* expression) in full strength salinity, European eel pre-leptocephalus larvae reared at iso-osmotic salinity conditions seem to be able to utilize energy more efficiently to improve growth and survival.

Water transport

Ion regulation is tightly coupled to water flow across membranes, where aquaporins form pores to selectively facilitate rapid transport and exchange of water molecules (Agre, 2006). In adult European eel, branchial *aqp3* was downregulated, but intestinal *aqp3* was unchanged (Cutler et al 2007), while renal *aqp1*, *aqp1dup* and *aqpe* were downregulated (Martinez et al 2005), but intestinal *aqp1* and *aqpe* were upregulated (Martinez et al 2005 b) in relation to seawater acclimation. In the current study, irrespective of treatment, *aqp1dup* showed highest expression at larval hatch (0 dph) and significantly decreased with increasing age, probably revealing a minor involvement during the larval stage. On the other hand, and similar to previous observations in European eel larvae (Politis et al 2018a), expression of *aqpe* increased throughout ontogeny, revealing that the function of this gene increases with increasing age, most probably following the increasing functionality of associated organs. In contrast to both (*aqp1dup* and *aqpe*), the function of *aqp3* seems to be of basal importance throughout European eel early larval ontogeny, as expression levels were constant from hatch until the first-feeding stage. It is worth to mention, that as gills are not present during this life stage, the functionality of this mechanism correlates to the development of kidney and intestine, which are only starting to form throughout this investigated period. As such, the tissue specific expression of this molecular mechanism, the functionality in earlier (embryogenesis) and later (feeding) stages, but also the potential sensitivity shift among developmental stages in relation to salinity change need to be further clarified in future studies.

Stress and repair response

The hypothalamic-pituitary-interrenal (HPI) axis, consisting of the corticotropin-releasing hormone (CRH) and urocortins exerted by activating two types of distinct CRH receptors, is a molecular mechanism regulating internal homeostasis in response to external challenges and stressors (Grammatopoulos 2012). This mechanism, originally associated to osmoregulation but also driving a wide range of physiological effects, has been strongly conserved throughout vertebrate evolution (Lovejoy and Balment 1999) and has been described in several fish species such as chum salmon, *Oncorhynchus keta* (Pohl et al 2001), Japanese pufferfish, *Fugu rubripes* (Cardoso et al 2003), and common carp, *Cyprinus carpio* (Huising et al 2007). Moreover, early expression of CRH related actors suggest a function during embryogenesis in zebrafish (Alderman and Bernier 2009) and larval development in sea bass, *Dicentrarchus labrax* (Mola et al 2011). In our study, 2 subtypes of CRH receptors were identified in European eel and their expression generally increased towards the first-feeding stages, confirming the involvement and increasing functionality of this mechanism throughout early fish ontogeny. Furthermore, the expression of heat shock proteins (*hsps*) in response to thermal stress has previously been shown in larval delta smelt (Komoroske et al 2015) and linked to developmental failure (deformities) of green sturgeon, *Acipenser medirostris* larvae (Werner et al 2007). Overall, HSP function is a known cellular mechanism commonly associated but not restricted to thermal vulnerability and has been recognized to

have a more universal role in response to a number of stressors (Hsu et al 2015). For instance, *hsps* have also been shown to be sensitive to changes in salinity in Black sea bream, *Mylio macrocephalus*, where low levels were associated to iso-osmotic conditions and high levels to hyper- or hypo-osmotic conditions (Deane et al 2002). A similar response was observed in European eel, where *hsp90* levels decreased in larvae reared in salinity reduced conditions compared to full strength seawater (36 psu), suggesting that iso-osmotic conditions are less stressful in maintaining cellular homeostasis (Politis et al 2018a). In the current study, there is no uniform reaction of *crh* or *hsp* system in response to salinity changes, possibly due to a rapid habituation/desensitization of the eel larval endocrine stress axis as it has been described in Atlantic salmon (Madaro et al 2016). Similarly, stress increase (cortisol) started at around 15 min but returned to basal levels at 2 h following exposure to acute stressors in zebrafish (Pavlidis et al 2015), while downregulation of stress related actors at 4 h post stressor was observed in rainbow trout, *Oncorhynchus mykiss* (Moltesen et al 2016). As such, it is not clear when the larval eel endocrine stress axis is able to acclimatize and how long this adaptation mechanism needs, but adapting to the iso-osmotic salinity changes applied in this study seems to be within the physiological tolerance thresholds limits of the species. Moreover, the genotype (family) × environment (salinity) interaction observed for *hsp90* at mouth opening, translates into differential responses of each batch to the salinity treatments, revealing an individuality in sensitivity to environmental stressors. On the other hand, the expression patterns of CRH receptors were “family” driven and varied among larval batches, suggesting a maternal programming of progeny with respect to baseline and stressor-induced mediators of the HPI axis activity, as also previously observed in wild sockeye salmon, *Oncorhynchus nerka* (Sopinka et al 2017).

Immunology

Understanding the immune system is vital to improve fish health and survival under rearing conditions. As in all vertebrates, teleost fish possess an innate and an adaptive (or acquired) arm of immune response (Uribe et al 2011). Fish offspring, especially during the earliest life stages, are considered highly sensitive and largely rely on the innate arm of the immune response, as their immune system is still under development (Vadstein et al 2013). In some marine fish larvae, such as cod, *Gadus morhua*, it can take up to three months until their immune response is fully functional (Magnadóttir et al 2004). In Japanese eel, some immune factors (i.e. lectin) are present 8 dph, whilst the appearance of most immune organs occurs late during larval development (Suzuki and Otake 2000). In European eel, a delicate phase of immuno-sensitivity has been described during early larval development, revealing a useful toolbox of immune genes in this species (Miest et al 2019). In this study, utilizing this toolbox, a group of immune genes were targeted to elucidate processes of both, the innate and adaptive immune system in European eel larvae. As part of the innate immune arm, initial detection of antigens is among others carried out by toll like receptors, where the here targeted *tlr2* is known to be involved in the recognition of bacterial and parasitic ligands in fish (Samanta et al 2012). Cytokines then induce a cell-signaling cascade, leading to an inflammatory immune response after detection of pathogen and danger associated molecular patterns (Savan et al 2006). The here targeted expression of *il1β*, in particular, leads to activation of lymphocytes and synthesis of acute phase proteins and thus activation of the complement biochemical cascade (Murphy et al 2012). Thereafter, the major histocompatibility complex carries out presentation of antigens to adaptive immune cells (such as T-cells), initiating the adaptive immune

response and ultimately leads to the destruction of the pathogen (Murphy et al 2012). The here targeted *mhc2*, is known to be located on the cell surface of antigen-presenting cells (e.g. macrophages, B-cells), where it is suggested to recognize bacterial and fungal pathogens in eel (Callol et al 2013). All genes (*tlr2*, *il1b*, *mhc2*) were expressed already at hatch, indicating that eel larvae were probably already able to detect pathogens at this stage (presence of *tlr2*) with the potential to mount a basic pro-inflammatory immune response (presence of *il1b* and *mhc2*). However, this ability most probably increases throughout ontogeny, justified by the increasing expression patterns observed towards the mouth-opening and/or first-feeding stages irrespective of the rearing salinity. This would be in accordance with the immunocompromised window previously described during European eel larval ontogeny from hatch until the teeth-formation stage (Miest et al 2019). Nonetheless, the strong maternal driver observed in our results (based on the clear family effect on all immune genes) and the genotype × environment interaction observed in *mhc2* and *il1b* (at mouth opening and first-feeding, respectively), probably reveals that eel offspring can be genetically equipped with a stronger or weaker immuno-readiness to respond to pathogens especially under different environmental stressors. This would be in accordance with a previous study revealing that certain genotypes of *mhc2* were associated to higher survival of carp in response to infection (Rakus et al 2009). This might be of great importance for broodstock selection in future breeding programs for eel aquaculture and should be further explored. Interestingly, the expression of immune related genes has also been shown during European eel embryogenesis, confirming maternal transfer of immune related factors for an initial protection prior to the activation of the zygotic transcription (Kottmann et al 2020). As such, future investigations should further clarify the developmental functionality and timing of innate and adaptive immune response, but also if and how the immuno-readiness can be shifted towards earlier stages, possibly by microbiome steering as well as maternal and/or larval immunostimulation (Miest et al 2016; De Schryver and Vadstein 2014; Franke et al 2017).

Timing and functionality of key molecular mechanisms

The expression patterns of 33 genes were investigated over time, from hatch (0 dph) to the first-feeding stage (12 dph), to elucidate timing and functionality of targeted key molecular mechanisms, driving and/or documenting health and development throughout early eel ontogeny (Fig 3). Four different patterns were identified, where gene expression either significantly peaked at hatch (0 dph), mouth opening (6 dph), first-feeding (12 dph) or was unaffected by larval age/stage. Irrespective of treatment, two genes relating to water transport (*aqp1dup*) and skeletogenesis (*bmp2a*) showed highest expression at larval hatch and significantly decreased with increasing age. The same expression pattern was previously observed in European eel for the same aquaporin gene (*aqp1dup*) but also other genes relating to thyroid hormone metabolism (*thba*), growth (*igf2a*) and immune system ontogeny (*c3*, *igm*) (Politis et al 2017; Politis et al 2018a,c; Miest et al 2019). As such, this expression pattern potentially reveals that the function of those genes might be of primary importance during earlier development and thus have been already expressed during the embryonic stage and/or were potentially transferred maternally (through the yolk) to the offspring as previously described in European eel (Kottmann et al 2020). Moreover, 16-18 % of the genes investigated in this study significantly peaked around the mouth opening stage, while revealing a core group of genes, linked to the molecular mechanisms of neurogenesis

(*neurod4*, *ngn1*) and heart development (*nppb*, *nppa*). More specifically, *neurod4* and *ngn1* work together to influence neuronal differentiation (Morrison 2001) and play a key role in a wide range of developmental processes (Massari and Murre 2000), while *nppb* and *nppa* are the predominant natriuretic peptides, playing key roles in cardiovascular and osmoregulatory homeostasis in European eel but also other vertebrates (Inoue et al 2015). As such, this expression pattern of consistently (across treatments) reaching a peak on 6 dph and in most cases decreasing to basal levels again beyond that, demonstrates that those key processes of neuronal differentiation and cardiovascular development occur at this European eel larval stage, irrespective of environmental drivers (such as salinity) and largely coincide with the genetically pre-programmed timing of primary organogenesis. Furthermore, the majority of genes, relating to almost all key molecular mechanisms targeted in this study, peaked at the first-feeding stage, which was to some degree expected as this corresponds to a timing of organogenesis refinement (e.g. brain, liver, gastro intestinal tract, etc.) and/or specific functional tissue development (e.g. feeding apparatus, eyes, etc.), in order to ensure optimal transition from endogenous to exogenous feeding (Sørensen et al 2016; Butts et al 2016; Politis et al 2018b). On the other hand, another core group of genes, which were not affected by larval age and showed a consistent expression pattern over time in all treatments, were linked to the molecular mechanisms of energy metabolism (*atp6*, *cox1*), thyroid metabolism/deiodination (*dio1*, *dio3*), skeletogenesis (*bmp5*) and water transport (*aqp3*). The function of those genes and their associated mechanisms, seem to be of basal importance for development, metabolism, and homeostasis throughout European eel early larval ontogeny from hatch until the first-feeding stage.

Interrelationship between genotype and environment

For each genotype, phenotypic trait, and environmental factor, a different reaction norm can exist, resulting in an enormous potential of interrelationships between genetic and environmental factors (Pfennig et al 2010; Kelly et al 2012; Jarquin et al 2014). Typical genetic × environment interactions have been observed in several fish species and reviewed by Oomen and Hutchings (2015). In the current study, the VC analysis revealed differing reaction norms, regarding larval eel phenotypic traits (gene expression), of each genotype (family) to the environmental variable (salinity) investigated. More specifically, on 6 dph (mouth opening), the expression patterns of 12 genes (~39%) associated to stress/repair (*hsp90*), immune response (*mhc2*), neurogenesis (*neurod4*), deiodination (*dio2*), thyroid metabolism (*thaa*, *thab*, *thbb*), energy metabolism (*atp6*), skeletogenesis (*bmp2b*, *bmp5*), growth (*igf2b*) and ion regulation (*nkcc2b*) were driven by the family × treatment interaction. Similarly, on 12 dph (first-feeding), the family × treatment interaction controlled the expression patterns of 5 genes (~15%) associated to water transport (*aqp3*), immune response (*il1b*), thyroid metabolism (*thbb*), skeletogenesis (*bmp5*) and heart development (*nppb*). As such, the results of this study reveal a phenotypic plasticity to salinity, as each genotype can produce different phenotypes (in terms of gene expression) when exposed to different environments (salinity treatments). This translates into a variable sensitivity of each batch to salinity (but most probably also other environmental factors), which should be taken into consideration in future larval culture of this species, as uncontrolled variability in extrinsic (environmental) parameters might unnecessarily challenge the condition and development of some eel offspring and thus negatively impact larval survival and production efficiency.

Technical applicability – biotic and abiotic interactions

Part of the observed variability in reaction norms can possibly be attributed to the relationship between biotic and abiotic interactions occurring within a RAS system. Salinity fluctuations are known to influence the microbial community composition (Navada et al 2019) and can impact the nitrification process of biofilters, responsible for ammonia and nitrite removal in RAS units (Kinyage et al 2019). As such, the abiotic change in salinity applied in the current study, could have directly and indirectly, differentially impacted the RAS biofilter, system water, and eel larval microbiome composition as well as their interactions. Consequently, this could partly explain the observed differential reaction norms regarding physiological responses and the interlinked gene expression of the eel larvae from different family crosses. From a RAS functionality point of view, it was previously shown that there is no advantage of small compared to large steps of salinity modification, as long as there is adequate time allowing for appropriate adjustment, as ammonia oxidation capacity seems to be linearly correlated to salinity acclimatization time (Navada et al 2019). However, the salinity modifications applied in eel larvi-culture do not allow for long acclimatization periods, challenging the stability of each RAS unit. In Japanese eel aquaculture, where the eel life cycle has been closed, larvi-culture is based on flow-through water systems, which is economically and ecologically not a really viable, efficient and sustainable procedure regarding water management. Interestingly though, intentional “priming” of water systems, by preceding exposure to salinity changes, leading to alterations in microbial community compositions, can result in “trained” RAS systems, able to accommodate improved nitrification during future salinity challenges (Navada et al 2020). Even though not tested in the current study, this could be an interesting microbial management strategy and tool for addressing issues in eel larvi-culture. Nonetheless, the current study has explored the possibility of applying a “drastic” salinity reduction, where larvae are directly moved from one system at full strength seawater to another system at ~50% reduced salinity on 6 dph. Despite this obviously extreme challenge, there was no negative impact observed on eel larvae experiencing this drastic salinity change compared to eel larvae reared in full strength seawater (control).

Conclusion and future perspectives

To summarize, the current study elucidated the functionality and timing of key underlying molecular processes driving and affecting European eel larval development and performance under different salinity regimes. Here, a genetic (parental) programming of molecular mechanisms and intrinsic batch-specific sensitivity to extrinsic (environmental) drivers need to be considered in future eel farming, especially when breeding programs become a reality. We conclude that eel larvae experiencing a salinity reduction are able to use their energy reserves more efficiently, leading to increased growth and improved survival. However, emerging challenges such as the edematous state of the larval heart seem to be linked to the timing of functionality of underlying molecular mechanisms (for instance involved in cardiovascular development) and need to be further addressed in future studies. On the other hand, we show that technical difficulties for introducing salinity reductions in European eel hatcheries can be circumvented by applying “drastic” changes, directly moving larvae from full strength seawater to iso-osmotic conditions. As such, the current study revealed the applicability of an ecologically viable and economically efficient salinity reduction protocol for eel larvi-culture, requiring only two stable RAS units, “primed” at the desired salinity levels. However, the technically most efficient and biologically suited timing for applying this “drastic” salinity reduction needs to be addressed in future studies.

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