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Transition from endogenous to exogenous feeding in hatchery-cultured European eel larvae

Elisa Benini [a,*,1], Sofia Engrola [b], Sebastian Nikitas Politis [a], Sune Riis Sørensen [a], Anders Nielsen [a], Luis E.C. Conceição [c], André Santos [c], Jonna Tomkiewicz [a]

[a] *Technical University of Denmark, National Institute of Aquatic Resources, Kgs. Lyngby, Denmark*
[b] *Centre of Marine Sciences, Universidade do Algarve, Faro, Portugal*
[c] *SPAROS Lda, Área Empresarial de Marim, Lote C, Olhão, Portugal*

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The transition from endogenous to exogenous feeding is critical during fish early life, where appropriate feed availability and timing of initiation of feeding influence survival. For European eel (*Anguilla anguilla*), establishing first feeding culture is at a pioneering state, where successful production of larvae has recently enabled feeding experiments. In the present study, three diets and potential benefits of early feeding during the transition from yolk-sac stage to feeding larvae were explored, including molecular analyses of genes involved in digestive functions and growth. Three consecutive trials were performed using hatchery produced eel offspring. In Feeding regimes 1 and 3, expression of *npy* and *cck* (appetite regulation) was higher, while expression of *pomca* (food intake) was lower in non-prefed larvae, indicating increased fasting and higher starvation risk. In contrast, Feeding regime 2 led to the highest survival ever registered for European eel larvae i.e. 20% at 20 dph, in spite that prefeeding resulted in reduced survival rate during the endogenous feeding stage. This was associated with initial *hsp90* (stress/repair) upregulation in larvae receiving prefeeding, however, with subsequent downregulation during exogenous feeding. Notably, the growth related gh expression was higher in prefed larvae, indicating growth benefits of prefeeding. Likewise, prefeeding resulted in pomca as well as *try*, *tgl*, and *amyl2a* (digestion) upregulation, providing evidence of beneficial maturation of gut functionalities. Essentially, Feeding regime 2 demonstrated a continuous upregulation of growth, appetite and digestion related genes, which in combination with the highest survival suggest that dietary requirements were partially met. Moreover, in Feeding regime 2, *gh* and *tgl* were expressed at a higher level in prefeeding larvae than in the control, indicating that prefeeding might be advantageous in spite observed mortality, but further research is needed, including timing of feed application.

1. Introduction

One of the challenges of diversification and closed-cycle production in aquaculture is the establishment of feeding larval culture. Standardized larval culture procedures have been established for a range of aquaculture species, however survival rates are often low or variable, while growth potential in most cases is not fully utilized (e.g. Conceição et al., 2003, 2010; Valente et al., 2013). In this context, the European eel (*Anguilla anguilla*) is a high-value fish species with a great potential for aquaculture provided the establishment of hatchery technology (Tomkiewicz, 2019). While protocols for successful production of offspring have been realized, development of larval culture is challenged by gaps in knowledge regarding the ontogeny and physiology of the early life history stages, including the enigmatic feeding stage, i.e. the leptocephalus larvae. These gaps need to be filled in order to succeed in a closed-cycle production and sustainable aquaculture of this species.

In general, insufficient knowledge about nutritional requirements of first feeding fish larvae is among the key causes of high mortalities and quality issues commonly observed in larval culture (Hamre et al., 2013; Houde, 1972; Rønnestad et al., 2013; Sifa et al., 1987, Tocher, 2010). This is particularly evident during the transition period from endogenous (utilizing exclusively yolk reserves) to exogenous feeding

* Corresponding author.
E-mail address: elibe@aqua.dtu.dk (E. Benini).
[1] **Present address**: DTU Aqua, Kemitorvet, Building 202, 2800 Kgs. Lyngby, Denmark

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(exclusively relying on external feeds). During this period, fish larvae acquire the ability to feed, which involves a combination of morphological, behavioral, and physiological features (Yúfera and Darias, 2007). In addition, fish larvae start swimming and must actively search for prey, which they need to recognize and process (Kamler, 1992; Rønnestad et al., 2013). Thus, to successfully achieve the transition to exogenous feeding in culture, feeding regimes need to be tuned at the onset and timing of developmental events in the fish larvae. A delay and/or failure to establish successful first feeding may cause morphological deformities, abnormal behavior, and inability to swim or feed, leading to high mortality (Gwak and Tanaka, 2001; Kjørsvik et al., 1991; Rønnestad et al., 2013). In this regard, if feeding does not occur shortly after the exhaustion of yolk reserves, fish larvae tend to reach the point-of-no-return. Beyond this point, even if they ingest feed, the larvae may not be able to digest or assimilate and eventually perish (Kamler, 1992; Yúfera and Darias, 2007). Therefore, identifying the appropriate timing for introducing feed during larval development is pivotal to obtain robust animals at later developmental stages.

General recommendations claim that fish larvae should receive feed for the first time as soon as they achieve feeding ability, when the feeding and digestive apparatus is developed and functional or when larvae start to swim actively in search for feed (Zambonino Infante and Cahu, 2007). Still, a delay has been observed between the time when feed was offered for the first time and tangible active ingestion by the larvae in several fish species (Garcia et al., 2020; Gisbert et al., 2004; Lima et al., 2017; Mookerji and Rao, 1999; Zhang et al., 2009). For example, Northern anchovy, *Engraulis mordax,* larvae reach the point-of-no-return only 1.5 days after yolk sac depletion and larvae are not able to survive, if feed is not introduced timely (Lasker, 1970). Similarly, hatchery-reared silver therapon, *Leiopotherapon plumbeus,* have a very brief transitional feeding period (~12 h after yolk exhaustion), where initiation of feeding beyond this period results in 100% mortality (Garcia et al., 2020). Therefore, offering feed to larvae earlier than complete yolk sac exhaustion may be a strategy to ensure high performance of fish larvae.

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

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

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

For the European eel, first attempts to raise larvae on an enriched rotifer paste proved unsuccessful (Butts et al., 2016), however, increased capability to produce viable larvae reaching the feeding stage, has promoted progress towards first feeding culture of European eel larvae (Tomkiewicz et al., 2019). In this regard, a recent study investigating the endocrine regulation of feeding, during the transition from endogenous to exogenous feeding, identified the first feeding window and described a genetically pre-programmed feeding mechanism with molecularly early maturing digestive functions (Politis et al., 2018a). Here, eel larvae expressed genes related to digestion and appetite already at 4 days post hatch (dph), indicating an adaptive potential towards a prompt maturation of the gastrointestinal function. While development of European eel hatchery technology steadily progresses, no diets have yet proven effective for establishment of feeding larval culture (Tomkiewicz et al., 2019).

In the present study, three pioneering trials were performed to test effects of different diets and feeding regimes on ingestion and digestion capacity of hatchery reared European eel larvae. At the same time, the potential benefit of an early introduction of feed on larval performance and culture techniques has been explored. Thus, three feeding regimes applying four formulated diets were tested. Larval performance was assessed through biometry and survival as well as at the molecular level through expression patterns of genes relating to growth [growth hormone (*gh*) and insulin-like growth factor 1 (*igf1*)], appetite [cholecystokinin (*cck*), neuropeptide Y (*npy*), ghrelin (*ghrl*)], food intake [proopiomelanocortin (*pomc*)], digestion [trypsin (*try*), triglyceride lipase (*tgl*), amylase (*amyl2a*)], as well as stress [heat shock protein 90 (*hsp90*)] and immune response [interleukin 1 β (*il1β*)].

2. Materials and methods

2.1. Ethic statement

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

2.2. Broodstock management and offspring production

Female broodstock comprised silver eel obtained from a brackish lake, Saltbæk Vig (Zealand, Denmark), while male broodstock was raised from the glass eel stage at a commercial eel farm (Royal Danish Fish, Hanstholm, Denmark). The broodstock was transported to the EEL-HATCH facility (DTU Aqua, Hirtshals, Denmark). Upon arrival, the fishes were acclimatized for three weeks, before assisted reproduction protocols were implemented for gamete production according to Kottmann et al. (2020). In females, vitellogenesis was induced by weekly injections of salmon pituitary extract (Argent Chemical Laboratories, Washington, USA) at a dose of 18.75 mg/kg initial body weight, while final maturation was induced using 17a,20b-dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma-Aldrich Chemie, Steinheim, Germany) (da Silva et al., 2018; Kottmann et al., 2020). Male broodstock received weekly injections of human chorionic gonadotropin (Sigma-Aldrich, Missouri, USA) at 1.5 IU/g of fish (Perez et al., 2000). For each female, a pool of milt from 3 to 5 males was obtained, sperm density was assessed and standardized using a short-term sperm storage medium (Koumpiadis et al., 2021; Peñaranda et al., 2010).

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

Offspring abundance was estimated at 4, 24 and 48 hpf by calculating the number of eggs/embryos in 3 × 15 mL water samples collected from the incubators and extrapolated to match the total incubator volume. Estimated total numbers were used to estimate offspring production and embryonic survival (Benini et al., 2022). For assessment of batch hatching success, subsamples of ~ 100 embryos were randomly collected from the incubators at 48 hpf. The embryos were inserted into 200 mL sterile tissue culture flasks (VWR, Denmark) filled with seawater, including rifampicin and ampicillin (each 50 mg/L, Sigma-Aldrich, Missouri, USA) to counteract microbial interference (Sørensen et al., 2014). At ~12 h after hatch, the numbers of hatched larvae versus unhatched or dead embryos were recorded and hatching success calculated as number hatched versus the total numbers in the flasks.

2.3. Selection of larval batches for the experiment

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

2.4. Experimental rearing systems and stocking of larvae

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

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

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

2.5. Prefeeding and feeding procedures

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

At 10 dph, corresponding to the first feeding stage at ~20 °C (Politis et al., 2017), larvae were fed five times a day (at 2 h intervals), where diets were gently pipetted onto the bottom of each tank, at a concentration of 0.5 mL of diet per L of rearing water. Light was gradually increased (2 min) to intensity of 21.5 ± 3.9 μmol/m^2/s (Butts et al.,

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

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

[a] another 75.000 eggs were transferred to a parallel experiment

2016), while water flow was paused during feeding. Larvae were allowed to feed for 30 min, after which light was dimmed off (2 min) and water flow resumed. Following a feeding period, water in the tank was set to "flow-through" for 30 min to flush away the remaining uneaten feed. Thus, new clean water, adjusted to ~20 °C and 18 psu, was used to refill each RAS unit. Larvae were moved into clean tanks daily.

2.6. Diet formulation and composition

All diets were freshly prepared daily. Diet A was based on pasteurized hen egg yolk (Æggeblommer, Danæg, Denmark), fish protein hydrolysate (Diana-Aqua, France) as well as a small portion of copepod and shrimp hydrolysates. Diet B was prepared using pasteurized thornback ray (*Raja clavata*) egg yolk, deskinned krill extract (Akudim, Esbjerg, Denmark) and a small portion of soybean peptides (Sgonek Biological Technology Co. Ltd, China). The krill extract consisted of defrosted and deskinned krill, mixed with reverse osmosis water at a 1:2 ratio, sieved through a nylon mesh (0.2 mm mesh size) and heat treated for 30 min at 60 °C. Diet C was a mix of Diets A and B, including the same amounts of copepod and shrimp hydrolysates and similar amounts of thornback ray egg yolk, but reduced amounts of fish protein hydrolysates and krill extract. Diet D was similar to Diet C, including the same amounts of copepod and shrimp hydrolysates as well as the same reduced amounts of fish hydrolysates and krill extract, but now replacing ray with hen egg yolk. The proximal composition of the four diets is shown in Table 2. The fatty acid composition of the diets is included in supplementary table 1. Feed formulation details can be provided upon request, for non-commercial purposes.

2.7. Experimental design

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

2.7.1. Trial 1 – Feeding regime 1

The first trial applied Diet A at 0.05 mL of diet per L of rearing water during prefeeding (4–9 dph) and at 0.5 mL of diet per L of rearing water during feeding (10–20 dph). This feeding regime was formulated considering the consistent availability of stable high quality protein found in hen egg yolk as well as limited ability of fish larvae to digest native protein (Kolkovski, 2001). Thus, the inclusion of hydrolyzed proteins was hypothesized to improve survival and growth of eel larvae as demonstrated for other fish species (Gisbert et al., 2018). The diet contained 66% of protein and 22.6% of fat as described in Table 2, while the fatty acids profile can be found in the supplementary material.

2.7.2. Trial 2 - Feeding regime 2

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

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

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

2.7.3. Trial 3 - Feeding regime 3

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

2.8. Data collection

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

In order to estimate mortality, dead larvae were removed daily from each replicated tank and quantified. Larval survival was calculated based on cumulative daily mortality as a percentage on the initial number from 4 until 20 dph.

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

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

Expression of genes of all larval samples were analyzed in two technical replicates of each gene using the qPCR Biomark™ HD

<table>
<tr><th>Age (dph)</th><th colspan="2">Feeding regime 1</th><th colspan="2">Feeding regime 2</th><th colspan="2">Feeding regime 3</th></tr>
<tr><td>0
1
2
3</td><td colspan="2"></td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td>4
5
7
6
8
9</td><td>Prefeeding with diet A</td><td>No prefeeding</td><td>Prefeeding with diet B</td><td>No prefeeding</td><td>Prefeeding with diet B</td><td>No prefeeding</td></tr>
<tr><td rowspan="3">10
11
12
13
14
15
16
17
18
19
20</td><td rowspan="3">Feeding with diet A</td><td rowspan="3">Feeding with diet A</td><td rowspan="3">Feeding with diet B</td><td rowspan="3">Feeding with diet B</td><td>Feeding with diet A</td><td>Feeding with diet A</td></tr>
<tr><td>Feeding with diet C</td><td>Feeding with diet C</td></tr>
<tr><td>Feeding with diet D</td><td>Feeding with diet D</td></tr>
</table>

Fig. 1. Schematic overview of three European eel *Anguilla anguilla* larval feeding regimes (1–3) and treatments applied from 0 to 20 days post hatch (dph).

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

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

technology (Fluidigm) based on 48 × 48 dynamic arrays (GE chips). In brief, a pre-amplification step was performed with a 500 nM primer pool of all primers using the PreAmp Master Mix (Fluidigm) and 1.3 mL of cDNA per sample for 10 min at 95 °C; 14 cycles: 15 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 SSo-fast EvaGreen Supermix low Rox (Bio Rad) and DNA-Binding Dye Sample Loading Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 mM. The chip was run according to the Fluidigm 48 × 48 PCR protocol with a Tm of 60 °C. The relative quantities of target gene transcripts were normalized and measured using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Coefficient of variation (CV) of technical replicates was calculated and checked to be < 0.04 (Hellemans et al., 2007).

2.9. Statistical analyses

All data were analyzed using R studio statistical analysis software (Version 1.3.959, *RStudio: Integrated Development for R. RStudio, PBC, Boston, MA*). Residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted values) to ensure they met model assumptions. Data were log(10) transformed to meet these assumptions when necessary. Alpha was set at 0.05 for testing main effects and interactions. Treatment means were contrasted using Tukey's Honest Significant Difference test. Body area and oil droplet area as well as gene expression (15 genes) were analyzed using a series of mixed model ANOVAs (PROC GLM)(Brooks et al., 2017). The main model variables (treatment (control vs. prefeeding)) and age (0, 8, 13, 18 dph) were analyzed for every Feeding regime (1, 2 and 3), while replicated tanks were considered random. Moreover, the model included a random tank effect accounting for potential correlation of measurements taken within the same tank. The initial model tested, included an interaction effect between treatment and age. The model was reduced when possible. The final model was validated through analyses of the residuals.

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

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

where

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

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

3. Results

3.1. Survival

The survival of larvae (presented as mean ± SEM of the three

replicated tanks) per Feeding regime, from 4 to 20 dph, is illustrated in Fig. 2. Here, during the prefeeding period (from 4 to 9 dph), larval survival for control treatments was similar among batches, with mean values on 9 dph of 57.24 ± 9.38%, 60.38 ± 1.29%, and 58.6 ± 3.09% for Feeding regimes 1 – 3, respectively. Comparatively, survival in the prefeeding treatments was 40.48 ± 10.47%, 50.54 ± 2.28% and 57.99 ± 0.43% for Feeding regimes 1–3, respectively. The differences between the control and prefeeding treatments in Feeding regimes 1 and 3 were not significant, neither at the end of the endogenous (9 dph) nor within the exogenous feeding stage (Figs. 2A and 2C). However, in Feeding regime 2 (Fig. 2B), the survival of larvae at the end of the endogenous feeding stage (9 dph) was significantly higher in the control group compared to the prefeeding group ($p < 0.01$), however the effect diminished during the exogenous feeding phase and was not longer significant at 20 dph ($p = 0.276$). Interestingly though, the mortality probability was significantly increased ($p < 0.01$), when prefeeding was applied in the feeding regimes 1 and 2, while this was not detectable in Feeding regime 3 (Table 4).

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

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

3.2. Biometry

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

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

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

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

Regarding *gh* expression, a significant ($p < 0.01$) age × treatment interaction was detected in Feeding regime 1. Therefore, the effect of age at each treatment is illustrated in Fig. 4E and F, while the effect of treatment at each age is illustrated in Fig. 4G. In non-prefed larvae (control), expression of *gh* was significantly ($p < 0.01$) upregulated on 13 dph and remained stable until 18 dph (Fig. 4E), while for prefed larvae it was significantly ($p < 0.01$) upregulated on 13 dph and decreased at 18 dph (Fig. 4F). Consequently, in prefed larvae, *gh* expression was significantly ($p < 0.01$) higher on 13 dph, but significantly ($p < 0.01$) lower on 18 dph compared to non-prefed larvae (Fig. 4G). In Feeding regime 2, *gh* expression was significantly ($p < 0.01$) upregulated in prefed larvae (Fig. 4O) and significantly ($p < 0.01$) and continuously increased approximately 20000-fold throughout ontogeny, reaching peak values on 18 dph (Fig. 4N). In Feeding regime 3, a significant ($p < 0.01$) age × treatment interaction was detected. Therefore, the effect of age at each treatment is illustrated in Fig. 4W and X, while the effect of treatment at each age is illustrated in Fig. 4Y. Here, expression of *gh*, was significantly ($p < 0.01$) upregulated beyond 13 dph for both, non-prefed and prefed larvae (Fig. 4W and X). Consequently, *gh* expression was significantly ($p < 0.01$) higher in prefed compared to non-prefed larvae on 18 dph (Fig. 4Y). Markedly, the expression of *gh* was approximately 2-fold higher in Feeding regime 2

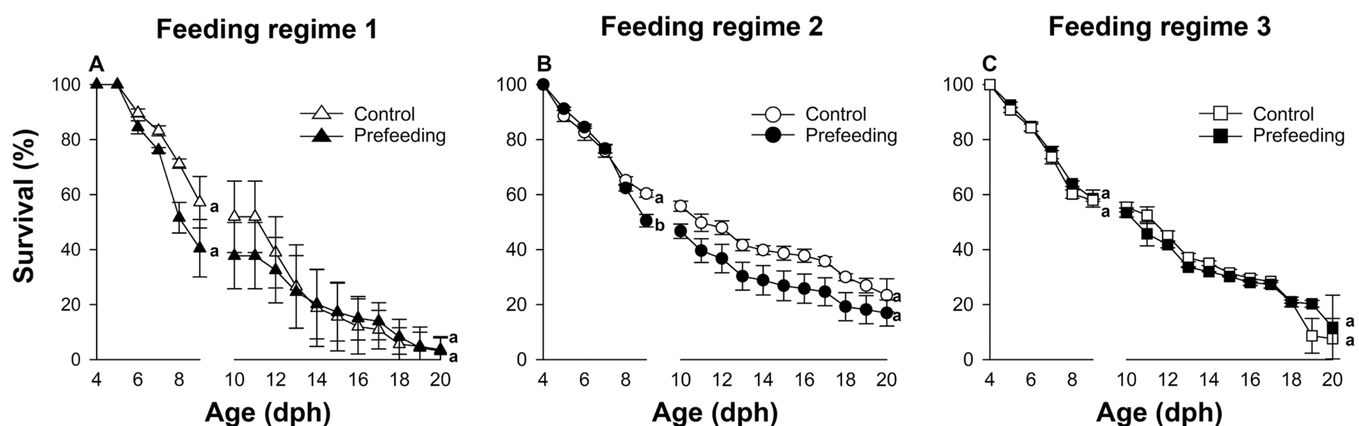

Fig. 2. European eel, *Anguilla anguilla* larval survival for three feeding regimes (1, 2 and 3) and two treatments (control vs prefeeding) from 0 to 20 days post hatch (dph). Values represent means (± SEM) of survival percentage, while values with different letters are significantly different at alpha = 0.05.

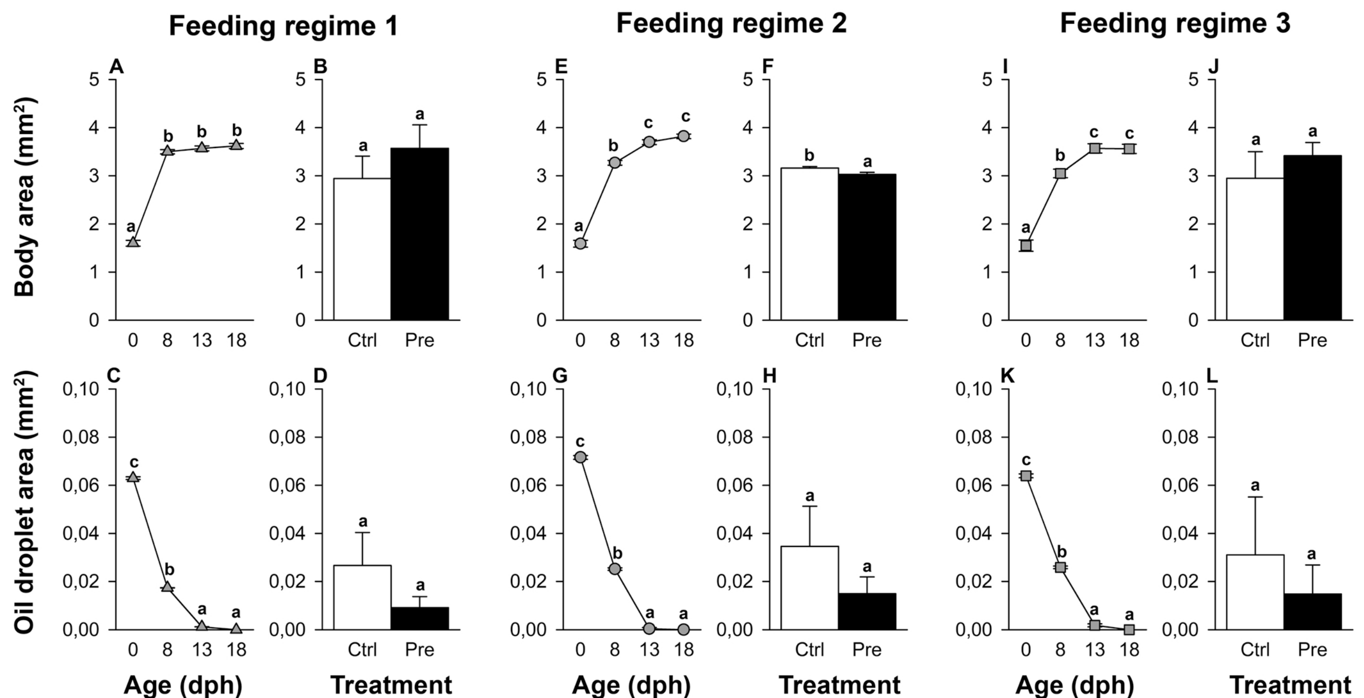

Fig. 3. European eel, *Anguilla anguilla* larval biometrics in three different feeding regimes. Measurements of body area (Feeding regime 1: A-B, Feeding regime 2: E-F and Feeding regime 3: I-J) and oil droplet area (Feeding regime 1: C-D, Feeding regime 2: G-H and Feeding regime 3: K-L). Effect of age in days post hatch (dph) and treatment (prefeeding (Pre) vs control (Ctrl)). Values represent means (± SEM) of body area and oil droplet area, while different letters represent significant differences at alpha = 0.05.

compared to the other Feeding regimes. On the other hand, *igf1* expression was not affected by treatment (Fig. 4I, Q, XY), but significantly ($p < 0.01$) upregulated on 8 dph in all feeding regimes (Fig. 4H, P, Z). Interestingly, *igf1* expression significantly ($p < 0.01$) decreased again beyond that point in Feeding regimes 1 and 3 but remained 2–3-fold higher upregulated in Feeding regime 2.

3.4. Appetite and food intake related gene expression

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

In Feeding regime 1, a significant ($p < 0.01$) age × treatment interaction was detected for the expression pattern of *cck*. Therefore, the effect of age at each treatment is illustrated in Fig. 5A and B, while the effect of treatment at each age is illustrated in Fig. 5C. Here, *cck* expression significantly ($p < 0.01$) increased on 8 dph and remained stably upregulated at this level until 18 dph for non-prefed larvae (Fig. 5A), while *cck* expression significantly ($p < 0.01$) increased throughout development and peaked at 13 dph but was downregulated again at 18 dph for prefed larvae (Fig. 5B). Consequently, *cck* expression was significantly ($p < 0.01$) lower in prefed compared to non-prefed (control) larvae on 18 dph (Fig. 5C). On the other hand, *cck* expression was not affected by treatments (Fig. 5L and T), but significantly ($p < 0.01$) increased continuously until 13 dph (Fig. 5K and S) in Feeding regimes 2 or 3. Interestingly, beyond that point, *cck* expression remained stable until 18 dph in Feeding regime 3 (Fig. 5S), while it continued to rise in Feeding regime 2 (Fig. 5K), reaching an almost 2-fold higher expression compared to the other Feeding regimes.

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

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

3.5. Digestion related gene expression

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

In Feeding regime 1, a significant ($p < 0.01$) age × treatment interaction was detected for the expression patterns of *amyl2a* and *try*. Therefore, the effect of age at each treatment is illustrated in Fig. 6A, B and Fig. 6F, G, respectively, while the effect of treatment at each age is illustrated in Fig. 6C and Fig. 6H, respectively. Here, *amyl2a* and *try* expression levels significantly ($p < 0.01$) increased on 13 dph and remained stably upregulated at this level until 18 dph for non-prefed

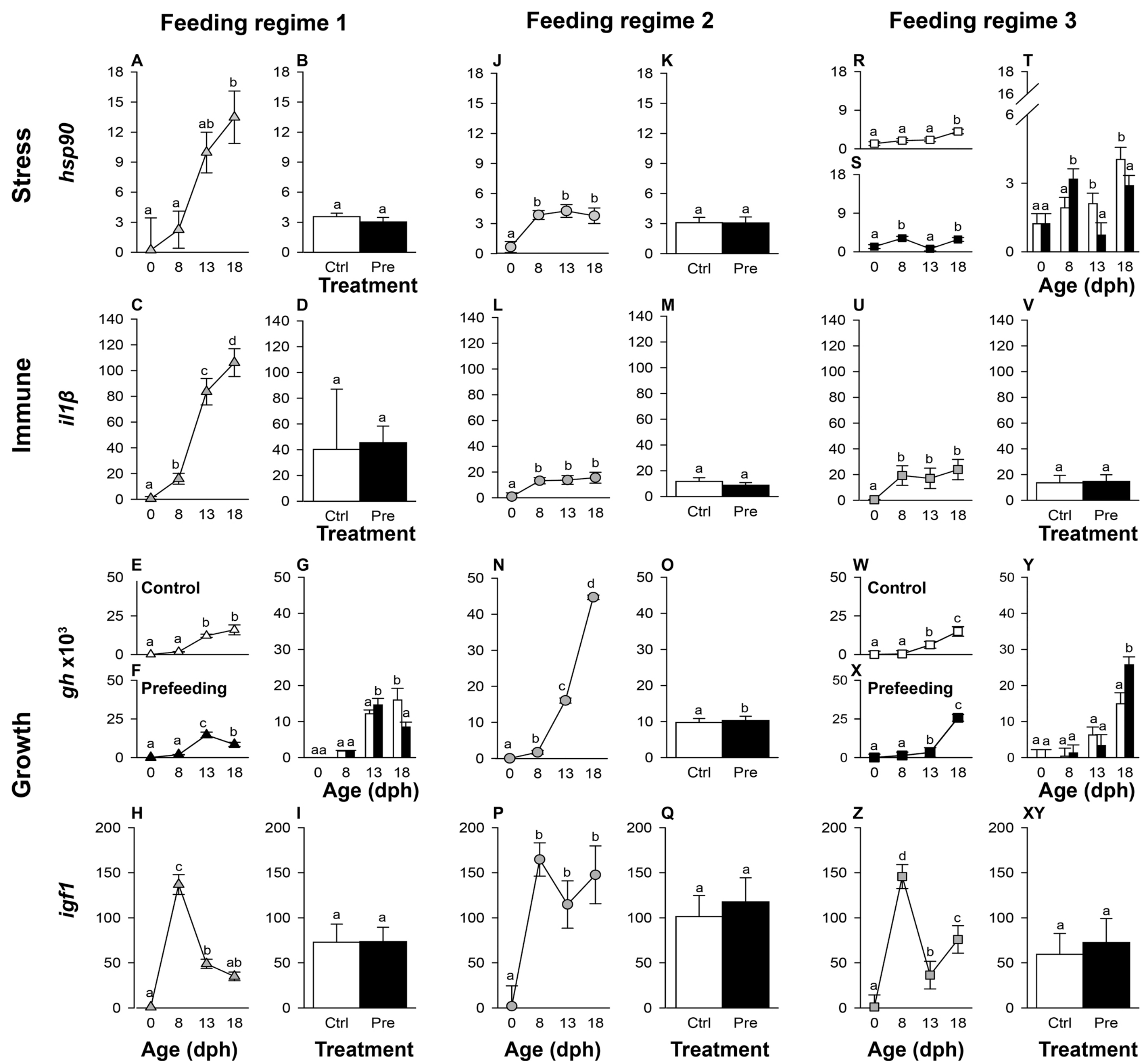

Fig. 4. Effect of age in days post hatch (dph) and prefeeding treatment (control (Ctrl) vs. prefeeding (Pre)) on European eel, *Anguilla anguilla* larval stress, immune and growth-related gene expression in three different feeding regimes (1, 2 and 3). Relative expression of heat shock protein 90 (*hsp90*),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

larvae (Fig. 6A and F), while *amyl2a* and *try* expression levels significantly ($p < 0.01$) increased at 13 dph but were downregulated again at 18 dph for prefed larvae (Fig. 6B and G). Moreover, *amyl2a* and *try* expression levels were significantly ($p < 0.01$) higher in prefed compared to non-prefed (control) larvae on 13 dph (Fig. 6C and H). On the other hand, *tgl* expression was not affected by treatments (Fig. 6E), while showing a significant ($p < 0.01$) 12-fold increase throughout the exogenous feeding period (Fig. 6D).

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

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

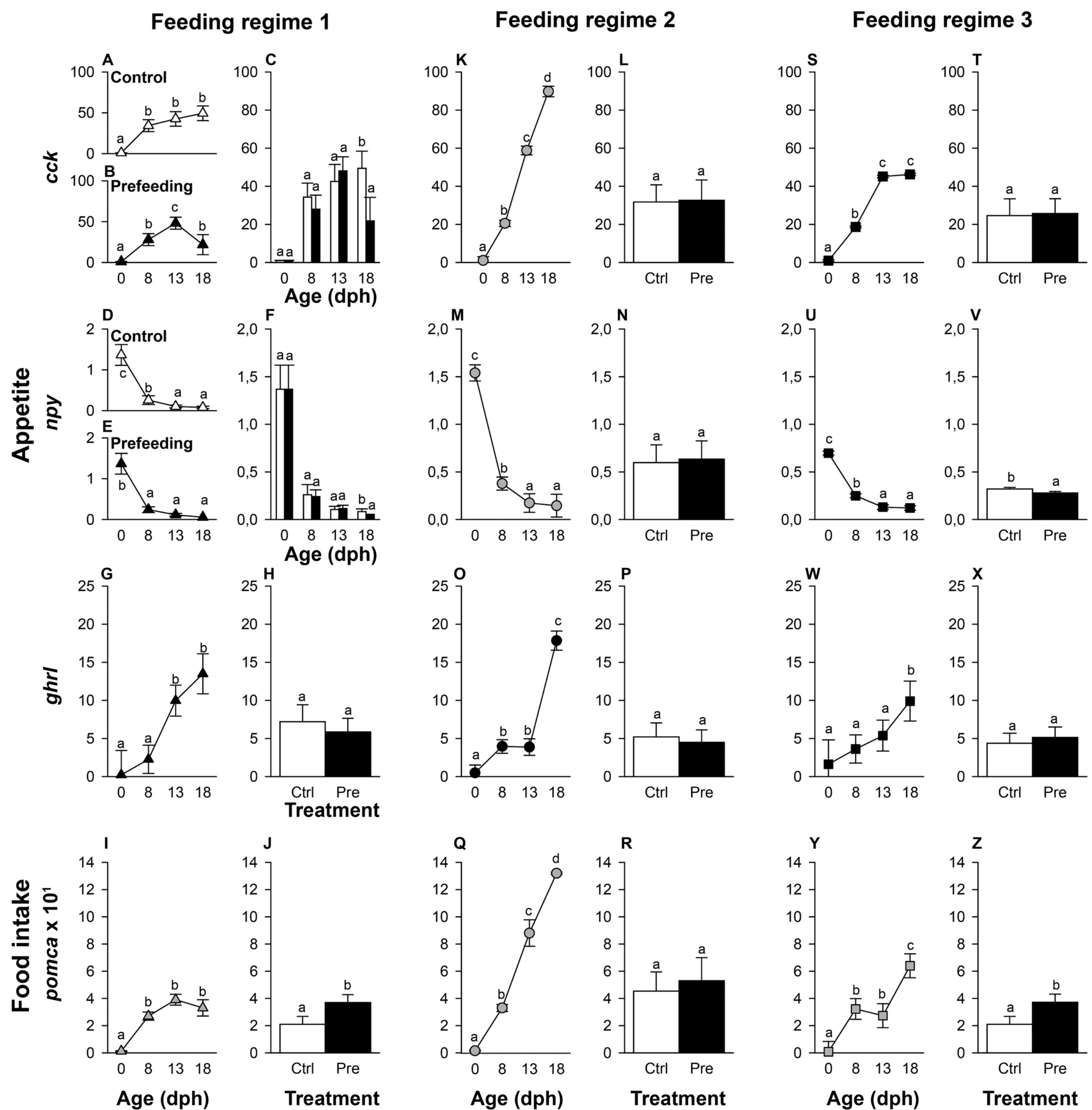

Fig. 5. Effect of age in days post hatch (dph) and prefeeding treatment (control (Ctrl) vs. prefeeding (Pre)) on European eel, *Anguilla anguilla* larval appetite and food intake related gene expression in three different feeding regimes (1, 2 and 3). Relative expression of cholecystokinin (*cck*) neuropeptide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

4. Discussion

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

4.1. Survival

Feeding regime 2 resulted in the highest survival ever registered for European eel larvae (~20% at 20 dph), but the early introduction of feeding reduced larval survival rate. Here, during the endogenous feeding stage, larvae receiving prefeeding had a lower survival compared to the control. However, no difference in survival was noticed at the end of the experiment. High mortality rates during the larval stage are commonly registered in nature as well as in aquaculture, despite the absence of predators, the environmental stability and the constant food availability providing better survival conditions (Peck et al., 2015). This

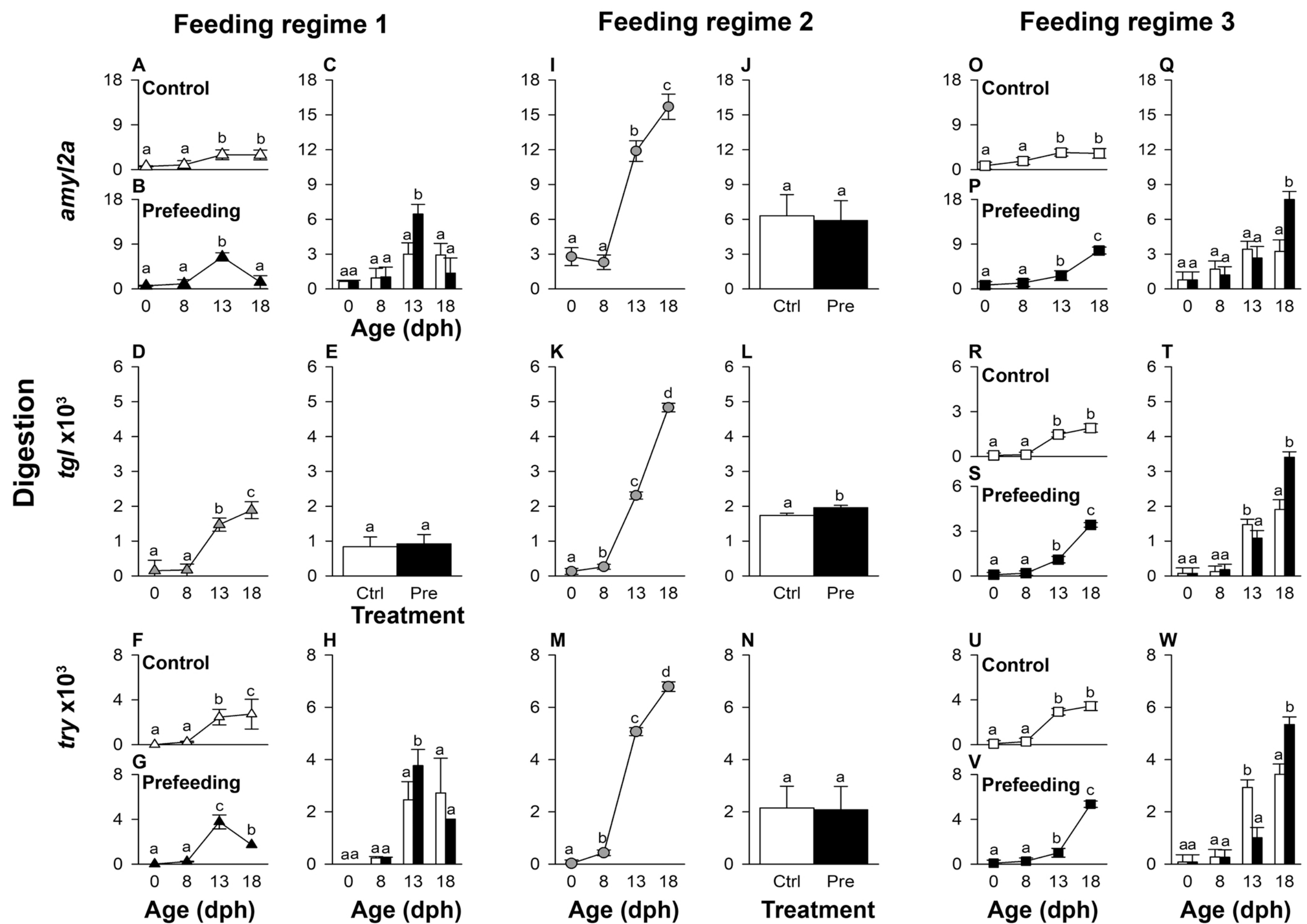

Fig. 6. Effects of age in days post hatch (dph) and prefeeding treatment (control (Ctrl) vs. prefeeding (Pre)) on European eel, *Anguilla anguilla* larval digestion related gene expression in three different feeding regimes (1, 2 and 3). Relative expression of amylase (*amyl2a*), triglyceride lipase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

is, to a large extend, connected to morphological and physiological changes, and often related to successful or unsuccessful molecular responses in the quest to adapt to new challenges (McMenamin and Parichy, 2013). In this regard, the early introduction of feed seems to challenge the larvae during a very sensitive period of their life, when the immune response as well as gut functionality are still under development. As such, while for some larvae getting in contact with feed (and the associated microflora) for the first time does not necessarily provoke any adverse physiological changes, for some other, with a different phenotypic responsiveness, prefeeding can be more challenging and possibly lead to mortality.

4.2. Stress/repair response

The expression of *hsp90* is considered a reliable indicator of the stress/repair mechanism activated by external stressors, as also described by Cara et al. (2007). Results from our study showed that after the initial *hsp90* upregulation, driven by the early introduction of feed, prefed larvae showed a repairing capacity demonstrated by the downregulation at 13 and 18 dph compared to non-prefed larvae (Feeding regime 3). However, stress during early life history can compromise development, as stressed larvae spend energy to restore and/or maintain homeostasis, which is then not further available to be invested into growth (Guderley and Pörtner, 2010). As such, in the current study, the potential stress caused by early introduction of feed might have affected eel larval growth as observed for larvae receiving prefeeding in Feeding regime 2. This is in contrast to studies on silver catfish, *Rhamdia voulezi* (Lima et al., 2017), obscure puffer, *Takifugu obscurus* (Shi et al., 2010) and Senegalese sole, *Solea senegalensis* (Engrola et al., 2009) larvae, where earlier feed introduction resulted in improved growth. Therefore, larvae offered prefeeding may be "primed" and show higher adaptability to new challenges, possibly by being better prepared to accept a full-scale feeding regime, but suboptimal procedures might cause high levels of stress during the transitional period between endogenous and exogenous feeding, leading to impaired growth. In this regard, it is possible that the introduction of prefeeding as early as 4 dph, when the eel larvae are undeveloped, could have provoked the observed stress reaction and, thus, a slight delay in application timing should be considered.

4.3. Growth

In the present study, growth related *gh* expression was upregulated in prefed larvae right after the prefeeding stage (on 13 dph) in Feeding regime 1, towards the end of the first feeding window (on 18 dph) in Feeding regime 3 and during the entire period in Feeding regime 2. Moreover, *igf1* expression, which was approximately 100-fold upregulated on 8 dph in all feeding regimes, decreased during the exogenous feeding period in Feeding regimes 1 and 3, but remained 2–3-fold higher upregulated in Feeding regime 2. Thus, the diet used in Feeding regime 2, seems to have partially met the dietary requirements of eel larvae and that the prefeeding principal has stimulated a molecular signal for

growth potential, which, however, was not translated into morphological growth performance.

4.4. Appetite and food intake

Larval growth also relies on mechanisms and processes relating to appetite and feed intake (Kestemont and Baras, 2001). In the present study, appetite related *ghrl* was not affected by prefeeding, but in non-prefed larvae *npy* was upregulated throughout the entire period in Feeding regime 3, while *npy* and *cck* were upregulated towards the end of the first feeding window (on 18 dph) in Feeding regime 1. In this regard, the expression of *npy*, which acts as an appetite stimulator (orexigenic factor), is commonly associate to fasting (Assan et al., 2021), while *cck*, which acts as an appetite inhibitor (anorexigenic factor), tends to be downregulated when the gut is full and upregulated when it is empty (Tillner et al., 2013). On the contrary, feed intake related *pomca*, which was proven to be a good biomarker to demonstrate feed intake in eel larvae (Politis et al., 2018b), was in the present study downregulated throughout the entire period for larvae not receiving prefeeding in Feeding regimes 1 and 3. Consequently, the higher expression of *npy* and *cck* as well as the lower expression of *pomca* observed in non-prefed larvae in the present study, probably indicate a likelihood of fasting and higher starvation risk. As such, we here provide evidence that the introduction of feed before mouth opening can influence appetite and feed intake related mechanisms in eel larvae, promoting the importance of the prefeeding principle.

4.5. Digestion

The transcription of genes encoding the major digestive pancreatic enzymes, such as trypsin, lipase, and amylase (overviewed in Fig. 7A, B, C), increased from basal levels during the endogenous feeding period to peak levels during the exogenous feeding period, confirming the molecular transition into the first-feeding window. This is a process, which is typically connected to genetically pre-programmed mechanisms related to digestion (Politis et al., 2018b), but can also be influenced by dietary composition (Zambonino Infante and Cahu, 2007). In the present study, prefed larvae showed upregulated *tgl* expression throughout the entire period in Feeding regime 2, upregulated *try* and *amyl2a* expression at 13 dph in Feeding regime 1, as well as upregulated expression of all three digestion related genes (*try*, *tgl*, *amyl2a*) at 18 dph in Feeding regime 3. Therefore, the earlier introduction of feed increased the production of digestive enzymes, thus, probably supported the maturation of the gastro-intestinal tract and prepared larvae to digest and assimilate nutrients. Furthermore, the expression levels of *try* were generally much higher compared to *tgl* and *amyl2a*, confirming the eel larval nutritional necessity for protein during this life stage, as previously described for European and Japanese eels (Hsu et al., 2015; Politis et al., 2018b).

4.6. General consideration about diets and feeding regimes

Overall, we could recognize general patterns of gene expression as shown in Fig. 7. As such, in Feeding regime 1, the standardized expression of genes relating to growth (*gh, igf1*), appetite (*cck*), and digestion (*try*, *amyl*) showed a similar pattern (Fig. 7A), where transcription unexpectedly "dropped" beyond 13 dph in prefed larvae. Here, the diet used in Feeding regime 1, which was based on hen egg yolk, seemed to be less attractive for European eel larvae to successfully initiate feeding and thrive throughout the first-feeding window. Moreover, the standardized expression of *il1β* and *hsp90* (Fig. 7D, E and F), was 4-fold higher in Feeding regime 1 compared to the others, revealing an immune and stress/repair response, potentially indicating the unsuitability of this Feeding regime. On the contrary, larvae in Feeding regime 2 showed a continuous upregulation of growth (*gh, igf1*), appetite (*cck*), and digestion (*try*, *tgl*, *amyl*) related genes, which in combination with the highest recorded survival values, indicate a positive effect of this Feeding regime. Here, Diet B used in this Feeding regime had a

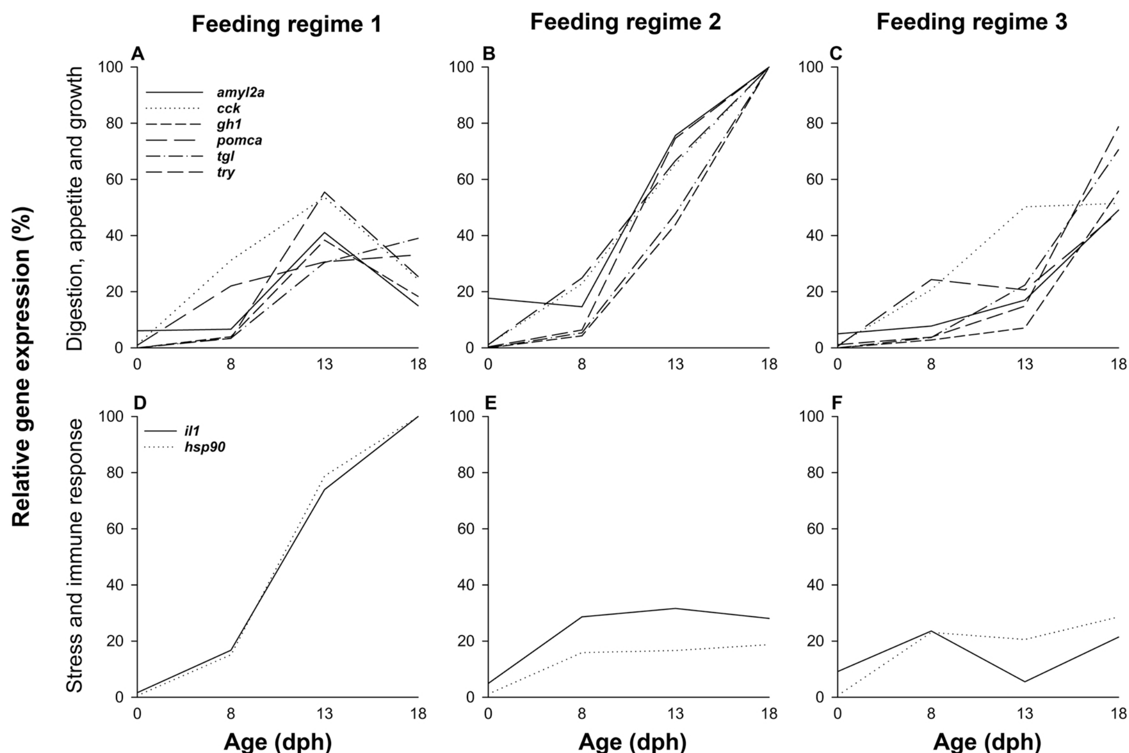

Fig. 7. Standardized expression of genes related to digestion, appetite, food intake and growth (A, B and C) as well as immune and stress response (D, E and F) in three different feeding regimes for prefed European eel, *Anguilla anguilla* larvae. Expression was calculated in relation to the highest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

much higher inclusion of protein (75%) and a much lower amount of lipid (12%) compared to Diet A and D (used in Feeding regimes 1 and 3), where it was 66% and 50% for protein and 22.6% and 40% for lipid, respectively. Thus, the composition of Diet B seems to be more adapted towards eel larval requirements, but the feed formulation and dietary regime still need to be further developed and improved for future larviculture of European eel.

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

5. Conclusions

In conclusion, feeding European eel larvae with Diet B in Feeding regime 2, demonstrated a continuous upregulation of growth (*gh, igf1*), appetite (*cck)*, and digestion (*try*, *tgl*, *amyl*) related genes, reaching values several-fold higher than in the other Feeding regimes. Moreover, this Feeding regime has led to a 20% survival at 20 dph, which is unpreceded in the quest to establish a European eel culture, pointing towards a dietary composition that approach the nutritional requirements of first-feeding eel larvae. Nevertheless, the feed formulation and dietary regime need to be further developed and improved for future larviculture of this species.

Additionally, the early introduction of feed (prefeeding) resulted in downregulation of appetite related *npy* and *cck,* but also upregulation of food intake related *pomca* as well as digestion related *try*, *tgl*, and *amyl2a*. Therefore, we conclude that early introduction of feed supported the maturation of the gastro-intestinal tract functionality, equipping the larvae with an improved digestive capacity. However, a slightly later application timing needs to be considered to potentially improve survival during the endogenous period, while at the same time maintain gut-priming benefits. At the same time, prefeeding triggered a molecular signal for growth potential, based on the upregulation of *gh*, but none of the feeding regimes seemed to provide a sufficiently balanced diet leading to biometrical larval growth, thus masking any potential initial benefits of prefeeding. In this regard, future eel dietary development needs to take levels of hydrolyzed proteins into consideration, especially considering stage specific requirements and/or preferences.

CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Agreement

All authors have seen and approved the final version of the manuscript that was hereby submitted. This article is the authors' original work, which has not received prior publication and is not under consideration for publication elsewhere.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2022.101159.

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