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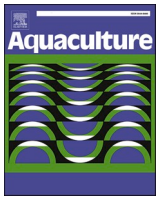

The role of hatching enzyme in European eel (*Anguilla anguilla*): Transcriptomic and enzymatic analysis after fertilisation and before hatching

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ABSTRACT

Teleost embryos utilise conserved zinc-binding astacin proteinases for the chemical modification of the glycoproteins composing the *zona radiata*. European eels, an evolutionary basal catadromous species, have multiple isozymes with similar catalytic functions for this task. Researchers are focusing on developing effective rearing practices for this critically endangered species as assisted reproduction efforts are currently obtaining variable embryonal hatching and survival. Thus, the objective was to investigate whether the variant hatching rate could be due to lacking high choriolytic enzyme 1-like, eel hatching enzyme expression and/or it's collagenase-like activity, while examining also the regulatory intercellular Zn^{2+} concentrations in developing embryos after fertilisation and pre-hatching. Six assisted reproduction was conducted for the collection of buoyant (vital) and sunk (non-vital) fertilised embryo pools two hours post-spawning (t0) and pre-hatching (t55). Real-time PCR was used together with Collagen degradation fluorometric assay, and Inductively Coupled Plasma-Optical Emission Spectrometry to investigate the intracellular Zn^{2+} concentrations. Transcripts of the eel hatching enzyme were detected in all sample pools with significant difference between vital and non-vital embryos even 2 h post-fertilisation, suggesting maternal derivation, or the onset of hatching enzyme expression before the known maternal-to-zygotic transition. Relative mRNA quantity depended on all factors (time, state, interaction) demonstrating an overall increase of expression. The collagenase-like activity was only time-dependent, and only non-vital samples contained higher intracellular Zn^{2+} concentrations than 0.6 mg kg^{-1}, suggesting a similar threshold as for other species. Correlation analysis revealed reverse correlations between Zn^{2+} concentration and the weight of the reproductive female at ovulation.

1. Introduction

Research efforts are focusing on the closure of the life cycle and the development of good practices used for rearing and assisting reproduction of European eels (*Anguilla anguilla*, Linnaeus, 1758), as the number in the local populations and the recruitment of the new generation glass eels from the Sargasso Sea has reduced drastically, and the species is now classified as "Critically endangered" by IUCN (International Union for Conservation of Nature) (Pike et al., 2020). The first feeding stage of European eel *leptocephalus* larvae has been obtained (Benini et al., 2023; Butts et al., 2016; Parmeggiani et al., 2020; Pfeiler, 1999; Politis et al., 2018), but in pursuit of closing the life cycle, the embryonal survival and hatching rates remain yet variable. Oviparous fish embryos have to chemically digest and break the chorion, the external layers of the egg envelop to start their larval stage. Hatching is one of the most critical moments directly impacting the quantity of the larvae, but it is also one of the most sensitive periods as the chemically modified chorion leaves the embryo less protected against external conditions, ultimately greatly impacting also the quality of the developing larvae. In contrast, unhatched embryos unavoidably undergo an

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apoptotic/senescence process (Jezierska et al., 2009; Yamanaka et al., 2025). Layers of the teleost chorion can be differentiated by their ultrastructure, origin and function, consisting of a thin outer *Zona pellucida* (ZP) layer and a thicker underlying *Zona radiata* (ZR) with its radial striations. The latter can be further divided as Chaudhry (1956) has demonstrated in *Trichiurus* and *Triacanthus* geni follicular origin of the ZP and the outer *zona radiata externa* while suggesting a partly follicular, partly ooplasm origin to the inner *zona radiata interna*. Both ZP and ZR layers consist of polymerized ZP proteins connected by non-covalent bonds creating a filamentous structure. The different ZP proteins commonly have an N-terminal region and a ZP domain. The latter consists of two folds (ZP-N and ZP-C) and a linker sequence between them (mid-ZPd) (Han et al., 2010; Litscher and Wassarman, 2018; Pérez-Atehortúa et al., 2023). In the teleost lineage, two genome duplication events created increased physiologic variability and resulted in a greater arsenal of genotypic, phenotypic, and behavioural strategies of the different species (Glasauer and Neuhauss, 2014), but commonly using hatching enzymes (HEs) for the enzymatic modification of the glycoproteins that compose the chorion (Korwin-Kossakowski, 2012; Pérez-Atehortúa et al., 2023). Teleost hatching enzymes belong to the astacin proteinase family, with a HExxHxxGFxHExxRxDR amino acid sequence (Pan et al., 2023). The sequence contains also a conserved zinc-binding motif (HExxH) constituting the active site of the enzyme (Inohaya et al., 1997; Yamagami, 1996; Yasumasu et al., 1992b), similarly present in other metalloproteinases like thermolysin, human fibroblast collagenase (McKerrow, 1987), or stromelysin (Whitham et al., 1986). From teleost species, HEs were first purified and studied in medaka (*Oryzias latipes*). Yasumasu et al. (1989a, 1989b) reported the presence of two different isoforms of "high" choriolytic enzymes (HCE-1 and HCE-2) and one "low" choriolytic enzyme (LCE). The HCE isoforms have 55% identity in amino acid sequence with LCE (Yasumasu et al., 1996), but the latter differs in its catalytic activity (Sano et al., 2014). During medaka hatching, HCE digests the N-terminal region of the ZP proteins, causing the swelling of the chorion, while LCE cleaves between the N-terminal region and the ZP domain, subsequently cutting further the ZP proteins by cleaving the linker sequence of the mid-ZPd. The cooperation of HCE and LCE is necessary for the complete digestion of the medaka chorion. In other commonly used model fish, like zebrafish (*Danio rerio*), similarly two enzymes have been revealed (Inohaya et al., 1997). Even at the genomic level, zebrafish genome encodes two HE (zHE1 and zHE2), but from a functional perspective, while zHE1 has a similar choriolytic activity as HCE, zHE2 is thought not to be necessary for the hatching (Sano et al., 2014; Small et al., 2020). Our understanding of HE in *Anguillid* sp., representing the evolutionary basilar *Elopomorpha* superorder, is based on studies conducted on Japanese eels (*Anguilla japonica*). Sano et al. (2011) reported multiple isozymes of eel-HE (EHE) with similar molecular weights and choriolytic function (same function as the HCE in medaka, or zHE1 in zebrafish). At genomic level, Hiroi et al. (2004) described a single multicopy gene, the high choriolytic enzyme 1-like gene (now on referred as eel hatching enzyme gene; *EHE*), from which transcripts were detected 20 hpf (hours post-fertilisation) at the anterior end of the forebrain with later expansion in number and distribution (26 hpf - 32 hpf) towards the yolk-sac until hatching (Hiroi et al., 2004).

Hatching enzymes are synthetized by the hatching gland cells (HGC) (Inohaya et al., 1999) in an inactive form (zymogens) with an autoinhibitory sequence at the N-terminal domain, which has to be cleaved for activation (Yasumasu et al., 1992b). Hatching gland cells are derived from the anterior end of the hypoblast layer (also known as the Polster), differentiating from the bipotential meso-endoderm cell population during the late gastrulation phase (Muraina et al., 2020; Okamoto et al., 2001). When HGC granules were isolated in the presence of EDTA, immunoblotting analyses revealed the inactive form of HCE and LCE (Yasumasu et al., 1992a). Otherwise, only the active form was detectable (Iuchi et al., 1982). This observation led to hypothesize autocatalysis, or the catalysis by another EDTA-sensitive metalloproteinase for the activation of HE (Yasumasu et al., 1992a, 1992b). Hatching enzymes have to bind Zn^{2+} cations to the active site to perform the catalytic activity, but the regulatory role of Zn^{2+} is already evident even more upstream throughout embryonal development. It is necessary for the migration and terminal differentiation of the HGC (Inohaya et al., 1997). The absence of Zip10, main intracellular importer of Zn^{2+} belonging to the Zinc/ Iron regulated transport (Zrt, Irt)-like protein (ZIP/SLC39) family (Huang and Tepaamorndech, 2013), results in isolated groups of HGC irregularly dispersed inside the embryo, and lead to transient expression of HE with major reduction of hatching rate (Muraina et al., 2020). On the other hand, multiple studies found that the concentration of zinc in the water is inversely related to the time of hatching, and excess waterborne zinc or zinc treatments also result in delayed and reduced hatching (Dave et al., 1987a; Küçükoğlu et al., 2013; Muraina et al., 2020).

European eels (*Anguilla anguilla*), reproducing in the Sargasso Sea, spawn type II marine pelagic eggs with visible oil droplets to acquire buoyancy (Cerdà et al., 2007; Heinsbroek et al., 2013; Korwin-Kossakowski, 2012; Tsukamoto et al., 2009). The buoyancy of the pelagic egg is an often-used indicator in controlled environment during artificial reproduction to assess the vitality, as the unfertilised oocytes and dead embryos will sink to the bottom of the tank (Kagawa et al., 2011; Pedersen, 2004; Sørensen et al., 2016). To reach sexual maturation in controlled environment, these animals undergo hormonal induction programs (Gallego et al., 2012; Herranz-Jusdado et al., 2019; Jéhannet et al., 2023; Mordenti et al., 2018; Palstra et al., 2005, 2022, 2023). However, the study of Pedersen (2004) revealed significant differences in embryonal development quality and its dependence on the hormonal regime of the spawner female silver eel. Therefore, the objective of the present study was to investigate if the variable hatching rates obtainable with the commonly used Carp pituitary extract (CPE) maturation-inducing treatment are due to any discrepancies in hatching enzyme mRNA content, or its' collagenase-like activity necessary for hatching. A secondary objective of the study was to investigate intracellular Zn^{2+} concentrations, acquiring preliminary insights into its intricate concentration range, which is necessary for normal embryonal development but does not result in reduced hatching rate.

2. Materials and methods

2.1. Animal handling

For the present study 6 assisted reproduction was performed with 6 female European eels after hormonally induced maturation. The Eels were originally caught in Valle Nuova (44°48′12″N – 12°15′16″E), a closed lagoon near the sluices of the North Adriatic Sea (Italy) during the autumn-winter migration period (November–February) towards the sea (Van Den Thillart et al., 2009). For the capture of the animals, the "lavoriero" (downstream trap) technique was used. Eels were anesthetized with a bath of clove oil (0.2 mL L^{-1}) on-site for biometric measurements to acquire the developmental silvering state (Durif et al., 2005a, 2006) as described (Casalini et al., 2023; Gentile et al., 2022). Then, transported in an insulated tank (300 L; approximately 2 h) to the facilities of Veterinary Medical Sciences (Cesenatico, Italy), where fish tags (FLOY TAG Mod Floy T-Bar Anchor) were applied in their dorsal muscle under anaesthesia with 400 ppm 2-phenoxyethanol (Mylonas et al., 2005). After measurement and tagging, the animals were placed in a recirculating tank system (RAS) with ambient conditions described in Mordenti et al. (2012) for a 10-day adaptation period prior to the hormonal induction program. The hormonal induction protocol consisted of weekly body weight (BW) control, and intramuscular injection of Carp Pituitary Extract (CPE; 5 mg kg^{-1} BW in the first four weeks, 15 mg kg^{-1} BW in the second four weeks, and 30 mg kg^{-1} BW from the ninth until full oocyte hydration) using the same anaesthetic protocol (Mordenti et al., 2018). Once the animal exceeded at least 110% of its initial BW, the protocol also incorporated ovary biopsy (every 24 h) with gentle pressure onto the abdomen from the cranial and caudal ends towards the

cloaca to control the oocyte hydration state under stereomicroscope for the timing of the ovulation induction (Mordenti et al., 2023a). The timing of the ovulation induction was set when at least 50% of the oocyte were in stage five development by Palstra et al. (2005), thus when the oocyte is fully transparent with the nucleus visible at the periphery, and the fat droplets are fused into a few large ones. To induce ovulation, intraperitoneal 2 mg kg^{-1} 17,20b-dihydroxy-4-pregnen-3-one (DHP) injection was made, and each ovulating eel was relocated separately for 12 h into a different seawater RAS (Mordenti et al., 2014) with four spermiating males at a temperature of 20 ± 0.5 °C to produce a thermic shock, which facilitates spontaneous spawning (Di Biase et al., 2016; Dou et al., 2008; Guarniero et al., 2020). In case of missing spontaneous spawning, gentle pressure was applied onto the abdomen from the cranial and caudal ends towards the cloaca to assist the spawning. All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (DIR. 2010/63/UE) and approved by the ethical committee of the Bologna University (ID 1157).

2.2. Sample collection

The first sampling (t0) was done two hours after spawning, as the oocytes have been released by the female, fertilised by the males, and the separation of the vital (with neutral buoyancy) and the sinking to the bottom of non-vital oocytes/zygotes has occurred. At this point, two pools of zygotes were collected until reaching 2 g weight for each pool from buoyant and non-buoyant ones accordingly. Then, the rest of the buoyant zygotes were transferred to incubator chambers described in Mordenti et al. (2014) with an approximated density of 250 zygote L^{-1}. Another sampling occurred as described 55 hpf (t55), when the first hatching larvae were observed. Similarly, 2 g embryo pools have been collected from vital-buoyant and non-vital/non-buoyant developing embryos. Each sample pool has been divided equally into 3 groups accordingly to the subsequent analysis, using metal free plastic tubes for the Zinc detection. Sample pools for collagenase-like activity and Zn^{2+} concentration analysis have been snap-frozen in liquid nitrogen and stocked at −80 °C until further analysis, as described below. The sample pools for transcription analysis were left in 1 mL RNAlater™ stabilization solution (Thermo Fisher Scientific, Waltham, MA,) overnight at room temperature. Then, after careful removal of the liquid phase, stocked at −80 °C until further analysis.

2.3. Reproductive performance: weight, buoyancy, fertilisation and hatching

For each reproduction, the following indicators (except hatching rate) were calculated in the first two hours after spawning. The weight of spawned oocytes (g) was calculated as the difference in female BW between post-spawning and the BW at the ovulation-inducing intraperitoneal DHP injection. Furthermore, the measurement of relative fecundity was also calculated as a percentile using the weight of the spawned oocytes divided by the weight of the animal at DHP injection and multiplied by 100 (Mordenti et al., 2023b). Buoyancy, fertilisation, and hatching rates have been obtained as described in Di Biase et al. (2017). Briefly, the buoyancy (or floating) rate of fertilised zygotes was obtained by maintaining a standardized quantity of samples for 30 min in a 500 mL beaker. After 30 min, the buoyancy percentile was determined by the number of buoyant zygotes divided by the total number put in the beaker and multiplied by 100. The fertilisation success rate of each reproduction was obtained by sampling standardized quantity of zygotes 2 hpf, and regardless of its buoyancy, counting the percentile of embryos reaching 8-cell or 16-cell stages. The hatching rate was calculated by sampling embryos and larvae in 1 L of incubation water between 55 h–60 h of incubation. Dividing the number of hatched larvae with the number of zygotes incubated and multiplying by 100 to obtain the percentage.

2.4. Total RNA extraction and qPCR for high choriolytic enzyme 1-like eel hatching enzyme

RNA extraction was performed using TRI Reagent (Molecular Research Center In, OH, USA) and NucleoSpin RNA II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) kit. Samples were homogenized in TRI Reagent (50–65 mg ml^{-1}) with an Ultra Turrax, then 200 μL of chloroform was added to the suspension and mixed well by vortexing. After incubation at room temperature for 10 min, samples were centrifuged (12,000 ×*g* for 10 min) and the aqueous phase was recovered. An equal volume of absolute ethanol (99%) was added, and the resulting solution was applied to the NucleoSpin RNA Column. RNA was purified according to the manufacturer's instructions. After spectrophotometric quantification, 250 ng of total RNA was reverse-transcripted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a final volume of 20 μL. To evaluate the transcript profiles, quantitative real-time PCR (qPCR) was performed using a CFX96 thermal cycler (Bio-Rad) with SYBR green detection for both the reference and the target high choriolytic enzyme 1-like (*EHE*) gene. The primers were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA) based on sequences available in the database (Table 1) (Parmeggiani et al., 2020). All amplification reactions were performed in 20 μL volumes and analysed in duplicates. The reaction contained: 10 μL of iTaq Universal SYBR Green Supermix (Bio-RAD), 0.8 μL of forward and reverse primers (5 μM each) for the target gene, 2 μL of cDNA and 7.2 μL of water.

The real-time program included an initial denaturation period of 1.5 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 30 s, then a melting step with ramping from 55 °C to 95 °C at a rate of 0.5 °C every 10 s. The relative quantification of mRNA content for the tested gene was evaluated using the ΔC_T value, where $\Delta C_T = (C_{T\ \text{mean reference gene}} - C_{T\ EHE})$, which directly correlates with relative expressions (Vandesompele et al., 2002). To compare expression changes between the sample groups in terms of Fold change, the $2^{\Delta\Delta Ct}$ method was used (Livak and Schmittgen, 2001) with the t0-non-vital group as internal calibrator.

2.5. Total protein and HE collagenase-like activity measurement

The total protein concentration was determined using a Protein Assay Kit (TP0300, Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. The obtained results were also used to normalise the measurements of collagenase activity. For the collagenase activity, a collagen degradation zymography test was conducted following the manufacturer's instructions (Abcam, Cambridge, United Kingdom). Samples were homogenized and incubated on ice for 5 min. Subsequently, the homogenate was centrifuged, and the supernatant was transferred into a fresh pre-chilled tube and kept on ice. Standards were prepared using 50 μM FITC standard, along with the assay buffer and added to a 96 well plate in duplicate to obtain the standard curve. The fluorescence was measured at Ex/Em 490/520 nm in end-point mode at RT. The sample lysate (50 μL, as verified in a preliminary assay) was added to the 96-well plate in duplicate. Afterwards, 50 μL of Collagenase substrate mix was added into each sample and the positive control well. Enzyme Positive Control (2 μL) was diluted with 18 μL of Assay Buffer and then 10 μL/well was used as suggested by the kit's instructions. The plate was mixed accurately, and the fluorescence was measured at Ex/Em 490/520 nm in kinetic mode for 2 h at room temperature as verified in a preliminary assay. Two time points were chosen for each sample where the corresponding RFUs were in a linear range, and Collagenase activity was calculated following the kit's instructions. The assay result was expressed as U/mg of protein where the Unit definition is the amount of collagenase required to cleave the collagen substrate and release 1 pmol of fluorescein per minute under the assay condition.

Table 1
The high choriolytic enzyme 1-like target gene (*EHE*) and reference genes (*40S, 18S, B-ACT*) with the specific primer sequences, base pair sizes, and accession numbers used for the relative quantification.

Gene		Primer sequence (5′→ 3′)	PCR size (bp)	Accession Number	Reference
EHE	For:	GGTGGCAAGCAGGTAGTGTCTC	174	XM_035408212.1	Present study
	Rev:	CTGAAAGTTGTAGATGGTGTGTGGTG		(NCBI)	
40S	For:	GTTCATCTTCAAGCCGTCCTGTG	143	GBXM01005349.1	Politis et al., 2018
	Rev:	TTGGTGAGGTTTGATCCGCATAATC		(GenBankTSA)	
18S	For:	GAGTCACGGAAGAGGATG	130	eeel2_s7245	Politis et al., 2018
	Rev:	TTAACAGGAGCCAGAAGAG		(EeelBase 2.0)	
B-ACT	For:	GGCTACTTCTTGTCATCGTTCTAC	101	DQ286836	Parmeggiani et al., 2015
	Rev:	TCGTAAGGCAGGCGTTCAC		(NCBI)	

2.6. Detection of intracellular Zn^{2+} concentration

Element analysis was performed using the Inductively Coupled Plasma - Optical Emission Spectrometry technique (ICP-OES) after microwave digestion of 0.7 g sample pool as described in Zaccaroni et al. (2014). Briefly, samples were microwave digested using a Milestone ETHOS ONE oven with 4 mL nitric acid. All reagents were obtained from Merck© (Darmstadt, Germany) with acids of "Suprapur" grade. Zinc ion concentrations were quantified by ICP-OES using a Perkin Elmer Optima 2100 DV instrument. Two blanks were run during each set of analyses to check for chemical purity. The accuracy of the method was verified with reference materials. All the values of the reference material were within certified limits. The instrumental detection limit, expressed as wet weight (w.w.), was 0.64 ng mL^{-1} with 90% recovery value. Zinc ion concentrations in the samples are expressed as μg g^{-1} on a w.w. basis.

2.7. Data analysis

For verifying the Gaussian distribution of the data and the variance, Shapiro-Wilk and Levene tests were used in R commander package of R (v4.3.2, R Core Team 2023, Vienna, Austria). Descriptive analysis was performed reporting the mean ± standard deviation (SD), minimum (MIN), and maximum (MAX) values for each group. Mixed-effect analysis for matched data, investigating also the interaction of the two factors (time and state), Sidak's multiple comparisons, Pearson correlation analysis, and graph creation were performed using GraphPad Prism (version 8.0.2). The significance level was set at $p < 0{,}05$ for all conducted tests.

3. Results

3.1. Reproductive performance

The six female eels (I-VI) enrolled originally weighted averagely 619.73 ± 78.36 g. Biometrical data indicated 4 female eels with silver index III (pre-migrant silvering eel) according to the classification of Durif et al. (2005b), while two were with silver index V (migrating silver eels). Previous studies have shown no significant difference in reproductive performance of pre-migrant female eels compared to the more advanced developing stages from the same territory (Casalini et al., 2024; Mordenti et al., 2023b). The animals were under CPE treatment between 18 and 23 weeks after which weighted 770.48 ± 106.74 g resulting in an average weight gain of 124.27 ± 6.72% compared to the initial body weight. At ovulation induction, the average weight of the animals was 830.02 ± 136.10 g, with a total weight gain of 133.42 ± 5.96% compared to the original. Three eels spawned spontaneously between 12 h–15 h after induction, while the others were assisted after 16 h, spawning a mean of 329.72 ± 81.60 g oocytes. This results to be an average of 39.40 ± 5.17% of the BW at ovulation induction (weight of female eel at DHP ovulation induction; FWDHP), after which the animals weighted averagely 500.30 ± 72.05 g. The percentage of spawned oocytes, buoyancy rates, fertilisation rates, and hatching rates are further reported by individual reproductive events (Fig. 1).

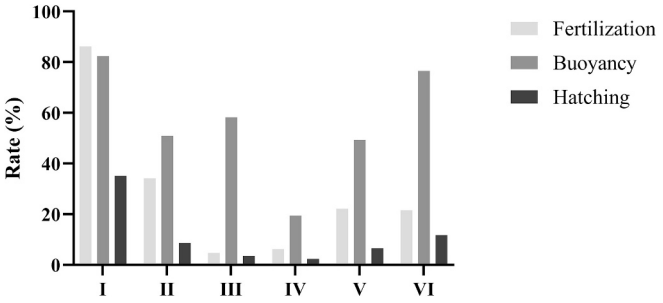

Fig. 1. Reproductive performance. Fertilisation, buoyancy, and hatching rates of the assisted reproduction of 6 female European eels after 18–23-week of maturation-inducing CPE program (I - VI).

3.2. Expression of eel hatching enzyme

All of the sample pools except one from the t55-non-vital group demonstrated transcriptome product of *EHE* with an observed melting point (Tm) at 81 °C. Hatching enzyme mRNA content was relatively lower both in the vital and non-vital groups at t0 compared to t55 (Fig. 2). Sidak's multiple comparisons resulted with significant difference between t0-vital/non-vital ($p = 0{,}0103$), t0/t55-vital (0,0016), t0-vital /t55-non-vital ($p = 0{,}0037$), while the difference between t55-vital and t55-non-vital does not result to be significant ($p = 0{,}8107$). For *EHE* mRNA content, the time ($p = 0{,}0009$), the state ($p = 0{,}0244$), as well as

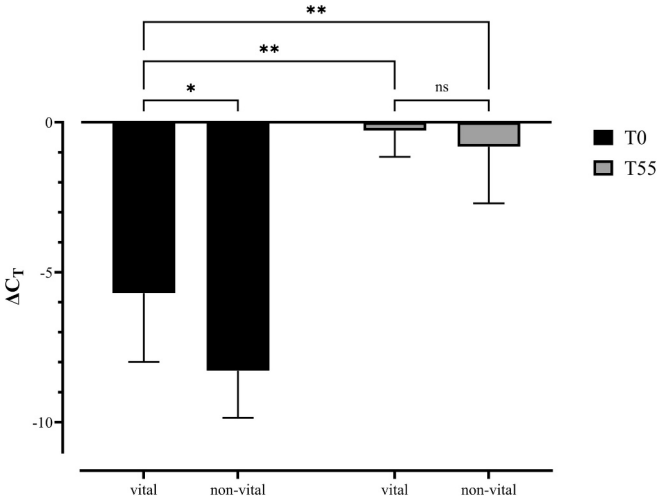

Fig. 2. Relative quantification of the high choriolytic enzyme 1-like target gene (EHE) mRNA content. Real-Time qPCR in buoyant (vital) and sunk (non-vital) European eel embryo samples after fertilisation (t0) and pre-hatching (t55). mRNA data were reported as ΔC_T (mean C_T of the 3 reference genes - C_T of target gene gene) * ($p = 0{,}0103$) and ** ($p \leq 0{,}01$) symbolize significant difference between groups. Less negative value indicates higher expression. Data are presented as mean ± SD.

their interaction (p = 0,0377) results to have significant effect. The change of mRNA content in terms of fold comparing to t0-non-vital group, which resulted to have the lowest *EHE* content and can include the difference of state between t0-non-vital and t0-vital, revealed an average 6-fold higher *EHE* content in vital embryos from the same sampling time-point, ranging between 1.40 and 25.51-fold (Fig. 3). Vital group at t55 results having 257.33 -fold higher expression compared to the t0-non-vital group, demonstrating the highest *EHE* expression in all groups. Non-vital embryos at t55 had 176-fold higher expression compared to non-vital embryos at t0, with great diversity ranging from 54-fold up to 571-fold, while the range of the vital embryos at the same time-point results to be more confined, between 147-fold and 449-fold.

3.3. Total protein content and HE collagenase-like activity

From the 24 pools, total protein contents were successfully measured for all. Only eighteen pools produced positive collagenase activity. The six pools with negative activity after the came from different reproduction rounds, specifically, two pools from the non-vital group at t0, one pool from the vital group at t0, one from t55-non-vital group, and two vital pools at t55. These samples have been excluded from descriptive and statistical analysis. Descriptive analysis performed on the protein content and collagenase activity of the samples grouped by the state and by the timing of the sampling (Table 2; Fig. 4). Non-vital zygotes at t0 had the highest total protein content on average.

Mixed-effect analysis of two-way ANOVA did not reveal any significant differences between the total protein content of the pools (State: p = 0,2916; Time: p = 0,3437; int: p = 0,1752), while a significant difference in collagenase activity was observed between the different time-points of sampling (p = 0,0418) with vital and non-vital samples having significantly higher average activity at t55 than at t0. Neither the state (p = 0,4613) nor the interaction of the two factors (p = 0,5688) had significant effects on the collagenase activity. Non-vital embryo at t55 had greater activity on average, but this difference did not result to be statistically significant, probably due to similarly high standard deviation in both groups at t55 (Fig. 4).

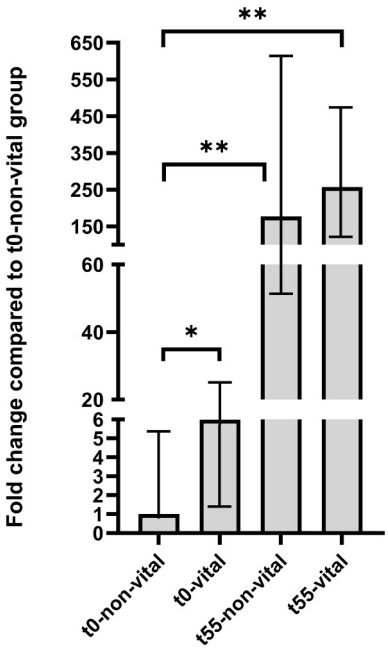

Fig. 3. Fold change of hatching enzyme transcripts in European eel embryos 2 h post-fertilisation (t0) and pre-hatching (t55). Calculated as fold of change ($2^{\Delta\Delta Ct}$) in relation to the non-vital embryo pool at t0 (ΔC_t interest group- ΔC_t t0-non-vital group). Data represent the mean ± the range of relative expression of six biological replicates per group. Sidak's multiple comparison post hoc test revealed significant differences of gene expression in the different groups (* p = 0,0103 and ** p ≤ 0,01).

3.4. Intracellular Zn^{2+} concentration

Intracellular Zn^{2+} was successfully measured for 23 of the total 24 pools. Specifically, the measurement of one pool from the t55-vital group produced a negative concentration, this result was excluded from descriptive and statistical analysis. Non-vital embryos at t0 contained the greatest average of intracellular Zn^{2+} cation concentration ranging from 0,0526 mg kg^{-1} up to 0,7530 mg kg^{-1}. Although no statistically significant difference was observable between any of the groups (neither for any of the factors), descriptive analysis reveals a lower intracellular concentration in average for the two groups at t55. Furthermore, in the vital groups at t0 and t55, the intracellular Zn^{2+} concentration did not exceed 0,6 mg kg^{-1}, while the groups of non-vital samples at t0 and t55 both had pools containing higher concentrations (Table 3).

3.5. Correlation analysis

The correlation analysis was conducted on 6 different reproductions and Spearman ρ values are reported for most of the measured parameters throughout this study (Fig. 5). The weight of the reproducing female eel at DHP injection (FWDHP) correlated positively, but not significantly to WSO (ρ = 0.7714; p = 0.1028), to the fertilisation rate (ρ = 0.7714; p = 0.1028), and to the collagenase-like activity of the t0-non-vital (ρ = 0.8000; p = 0.1333) group. Positive, but not significant correlation was found also with the activity of the t55-vital group (ρ = 0.5000; p = 0.4500), and the *EHE* mRNA content of the t55-non-vital group (ρ = 0.6000; p = 0.2417). Furthermore, FWDHP demonstrated strong negative correlation with the Zn^{2+} concentration of all groups: t0-non-vital (ρ = −0.7000; p = 0.2333), t0-vital (ρ = −0.9429; p = 0.0167), t55-non-vital (ρ = −0.8286; p = 0.0583), t55-vital (ρ = −0.7143; p = 0.1361). With the t0-vital group resulting to be significant. The total protein content also correlated negatively to FWDHP for the t0-non-vital group (ρ = −0.7142; p = 0.1361), and the t55-vital group (ρ = −0.8285; p = 0.05833). The weight of spawned oocytes showed positive correlation with the fertilisation rate (ρ = 0.8286; p = 0.0583), and hatching rate (ρ = 0.5429; p = 0.2972). With the collagenase-like activity of t0-non-vital (ρ = 0.6000; p = 0.3500), and t0-vital (ρ = 0.8000; p = 0.3333) groups. Negative correlation has been revealed between WSO and Zn^{2+} concentration for the t0-vital (ρ = −0.6571; p = 0.1750), t55-non-vital (ρ = −0.7714; p = 0.1028), and t55-vital (ρ = −0.7714; p = 0.1028) groups. The WSO also correlated negatively with the total protein content of the t0-non-vital (ρ = −0.9429; p = 0.0167), and t0-vital (ρ = −0.8857; p = 0.0333) groups, both resulting to be significant. Negative correlation was found for WSO also to the protein content of the t55-vital group (ρ = −0.8286); however, this latter does not result to be significant (p = 0.1361). The buoyancy rate demonstrated positive correlation with the hatching rate (ρ = 0.6571; p = 0.1750), and protein content of the t55-vital group (ρ = 0.6000; p = 0.2714), while negatively to the collagenase-like activity of the t0-vital group (ρ = −0.8000; p = 0.3333). Fertilisation rate correlated positively to the hatching rate (ρ = 0.8286; p = 0.0583), and collagenase-like activity of the t0-non-vital group (ρ = 0.5000 p = 0.4500), while negatively to the Zn^{2+} concentration of t0-vital (ρ = −0.8286; p = 0.0583), t55-non-vital (ρ = −0.7714; p = 0.1028), t55-vital (ρ = −0.9429; p = 0.0167) groups. The latter group being the only significant correlation for fertilisation rate. Hatching rate correlated negatively, but not significantly to the Zn^{2+} concentration of the t55-vital group (ρ = −0.7714; p = 0.1028). Many more positive and negative correlation has been found between the analysis of different sample pool groups, for which we invite the reader to consult the ρ values (Fig. 5), and the p values (Table S1) in supplementary material.

Table 2
Descriptive analysis of the total protein content and collagenase activity of European eel embryo samples divided by the different time points and states, and reported as the mean, standard deviation (SD), minimum (MIN), maximum (MAX), and number of samples (n) of the different groups.

	Protein (µg/µl)				Activity (U/mg)			
Time	t0		t55		t0		t55	
State	vital	non-vital	vital	non-vital	vital	non-vital	vital	non-vital
mean	1.60	2.30	1.72	1.63	19.37	23.89	62.51	97.43
SD	0.54	0.73	0.72	0.46	14.92	10.48	69.38	65.38
MIN	0.90	1.38	1.04	1.04	2.82	9.57	15.84	33.70
MAX	2.56	3.48	2.70	2.34	40.19	37.43	182.12	218.14
n	6	6	6	6	5	4	4	5

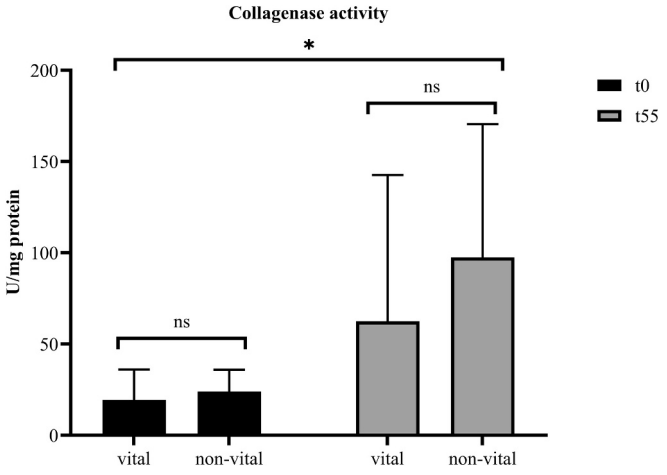

Fig. 4. Collagenase-like activity in vital (buoyant) and non-vital (sunk) European eel embryo samples after fertilisation (t0) and before hatching (t55). * Symbolize significant difference between groups of t0 and t55 ($p = 0{,}0418$), while ns symbolize non-significant difference.

Table 3
Descriptive analysis of European eel buoyant (vital) and sunk (non-vital) samples' intracellular Zn^{2+} concentration after fertilisation (t0) and before hatching (t55).

Zn^{2+} (mg kg^{-1})				
Time	t0		t55	
State	vital	non-vital	vital	non-vital
mean	0,3005	0,3894	0,2733	0,2041
SD	0,1427	0,2584	0,2088	0,2730
MIN	0,1034	0,0526	0,0214	0,0405
MAX	0,5389	0,7530	0,5560	0,8122
n	6	6	5	6

4. Discussion

In the present study, both *EHE* mRNA content and collagenase activity was dependent on the time factor, revealing an increased expression and activity of hatching enzyme in the later stages of embryonal development towards hatching. These results suggest rejecting the original hypothesis, the obtained variable hatching rates are not caused by discrepancy in the expression and/or the collagenase activity necessary for the chemical modification of the chorion. While looking at the reproductive performance, the conducted six reproductions underperformed compared to previous attempts (Mordenti et al., 2023b), mostly due to low percentiles of successfully fertilised zygotes. Nonetheless, the current study does not leave us without significant advancements regarding our understanding of teleost hatching enzymes, nor important considerations for the development of practices for the endangered species in object. Previously, the in situ hybridisation study of Hiroi et al. (2004) evidenced presence of hatching enzyme transcripts in 20 hpf, early neurula stage Japanese eel embryos. More recently, similar real time PCR measurement of He et al. (2017) evidenced upregulation of tilapia hatching enzyme transcription to start in gastrulation stage embryos. Lepage and Gache (1990) using sea urchin model demonstrated marked increase of hatching enzyme transcripts in cleavage stage embryos, but did not reveal hatching enzyme mRNA in unfertilised oocytes, concluding that hatching enzyme RNAs are not maternally deposited. In the present study, real time PCR revealed *EHE* transcripts already in 2 hpf embryos, downregulated compared to the average transcription of "housekeeping" gene, but with significant difference between vital and non-vital groups. Maternal mRNAs stored in the oocyte are crucial for regulating early embryonic development, specifically from fertilisation until the mid-blastula stage, when control is transferred to the zygotic genome. Considering only maternal deposition of mRNA in the mature oocyte, and possible implications of mRNA quantities onto the quality of cytoplasmic developmental processes (Fernández Míguez et al., 2024; Sullivan et al., 2015), the presence of hatching enzyme transcripts as soon as 2 hpf leads to different interpretations. It infers maternal derivation as Kottmann et al. (2020) suggests maternal-to-zygotic transition (MZT), the phase where embryonic mRNA transcripts take over accompanied by clearance of maternal RNA, to start only after 8 hpf for eel embryos. By this interpretation the results evidence the dependence of embryonal viability on the mRNA quantity deposited in the oocytes by the reproductive female eel. On the contrary, the non-vital state can also cause the degradation of the mRNA. The direction of causality is not clear, but the possibility of observing significant difference reduces the probability for the measured presence of *EHE* mRNA to be an artifact. Alternatively, similarly to the sea urchin hatching enzyme, *EHE* might have a unique feature to be expressed by the zygote prior then MZT. In this case, the non-vital state infers a missing activation of *EHE* expression, hence the significant difference found between vital and non-vital embryos at 2 hpf. This latter interpretation highlights the possibility for a different function of the hatching enzyme throughout the development of the embryo, but the current study does not provide direct evidence for neither interpretation, and further differences between sea urchin hatching enzyme and teleost hatching enzymes should not be neglected either. The increasing mRNA content in function both of time, state, and the significant interaction effect evidence an overall increase of embryonal *EHE* expression. The change of *EHE* mRNA content in terms of folds between the different time points gives us a superficial glance what could be the *EHE* content expressed by the embryo, but the big standard deviation in both t55 groups, and the uncontrolled depletion of maternal hatching enzyme mRNA (if any) prevents further considerations. Due to missing commercially available kit specific for hatching enzyme activity, a more general collagen proteolytic activity measurement resulted not to be dependent on the state, nor the interaction of the factors, but only on the time factor, reflecting an increasing activity along with the advancement towards the later embryonal stages. The greater activity in the sample groups at t55 evidences an overall greater choriolytic activity, but missing difference by the sample state (vital/non-vital) suggests further underlying intracellular mechanisms

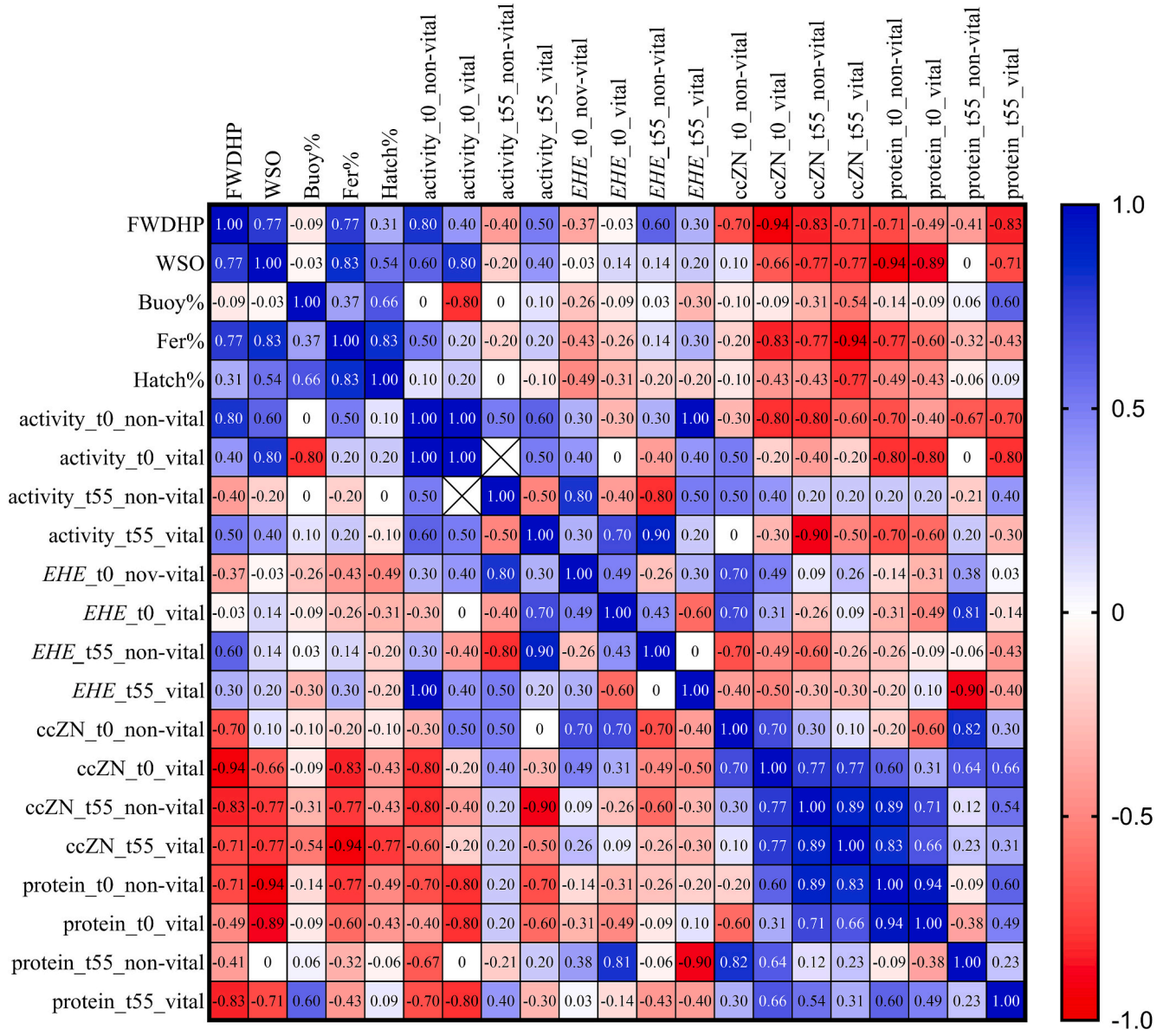

Fig. 5. Heatmap of Spearman correlation ρ values. Female eel weight at ovulation induction (FWDHP), weight of the spawned oocytes (WSO), Buoyancy rate (Buoy %), Fertilisation rate (Fer%), Hatching rate (Hatch%), intracellular Zn^{2+} concentration (ccZN), of buoyant (vital) and sunk (non-vital) European eel embryos after fertilisation (t0) and pre- hatching (t55). Crossed quadrats indicate not sufficient sample size for the analysis.

connected to collagen-like proteolysis. Different matrix metalloproteinases, like the MMP 1, MMP 8, and MMP 13 all have collagenase-like proteolytic activity (Pedersen et al., 2015) and associated to have higher expression and activity in apoptotic cells (Haschtmann et al., 2008; Segura-Valdez et al., 2000). Furthermore the serine-endoprotease zonase was also associated to have choriolytic activity (Aranishi and Nakane, 1997; Kristjánsson et al., 1995; Miftari et al., 2022). The activity of these, and other proteases with collagenase activity cannot be separated from EHE activity under the current experimental approach. The higher average collagenase activities in non-vital groups both at t0 and t55 hence can be attributed to caspases-activated apoptotic processes caused by hypoxia, developmental irregularities, or the impairment of hatching (Perry et al., 1997; Takle and Andersen, 2007; Truong-Tran et al., 2000; Yamashita, 2003). Nevertheless, to the best of our knowledge, this is the first report to quantify collagenase activity in eel embryos to provide useful insights for further analysis. While Zn^{2+} trace element is essential for many of the developmental processes, higher concentrations result to reduce the hatching rate. An earlier study by J. F. Skidmore (1964) suggests the chorion, and the perivitelline fluid to be the prime victims of zinc toxicity. Furthermore, Muraina et al. (2020) suggested zinc deposition or removal from the chorion, potentially making it harder or softer, in conclusion impacting the hatching efficiency. The ring test of Dave et al. (1987b) on zebrafish stabilized "no-effect" waterborne Zinc concentration at 0.5 mg Zn L^{-1}, while Küçükoğlu et al. (2013) studied the median lethal concentration value (LC_{50}) to be 0.65 mg L^{-1} for lone Zn^{2+}. Measured intracellularly, the current findings are in accordance with these previous studies as only non-vital groups contained analysed pools with greater than 0.6 mg kg^{-1} intracellular concentrations of Zn^{2+}, suggesting a similar threshold also for eel embryos. However, a more specified study would be necessary for the precise determination of an intracellular optimal range. Regarding the correlation analysis, while the low number of data repetitions certainly obscures many of the obtained correlations and *p*-values, considering the scarcity of information in regard we cared to share the findings, and the results should be considered accordingly. The numerous strong reverse correlations

between the Zn^{2+} concentrations with the fertilisation rate, and the weight of reproducing female eel remains intriguing.

One does not simply walk into the research of the teleost hatching enzymes. Its black gates are guarded by more than just confusing nomenclature. There are proteases there that do not sleep, and the phenotypical plasticity is ever watchful. Unfortunately, specific biochemical research evidencing causalities leading to the differences in ultra-structure between the ZP and ZR is not known. Although the consideration of the two different layers is adding to the complexity of research, it is important for the function specification of the HE. A recent study from Miftari et al. (2022) evidences further in *Masu Salmon*, direct contact of the ZP with the hatching fluid does not induce hatching underlining the notion that choriolysines, such as HCE, LCE, zHE, and EHE are not directly catalysing on ZP-proteins of the ZP, but the ZP-proteins of the ZR, causing the swelling and digestion of the latter during hatching (Hiroi et al., 2004; Inohaya et al., 1999; Yamagami, 1996; Yamagami, 1981), while the physical properties (thickness, composition), and the movement of the hatching larvae are also important factors determining the hatching efficiency (Korwin-Kossakowski, 2012; Sano et al., 2011). Ultra-structural changes during oocyte maturation in eels have been described both in Japanese eels (Adachi et al., 2003; Kayaba et al., 2001) and in European eels (Burzawa-Gerard et al., 1994; Gentile et al., 2022), but without particular attention to the changes of the chorion thickness. Considering the revealed positive correlation between chorion thickness and the number of maturation-inducing hormonal injection (Izumi et al., 2015), the aforementioned physical and dynamical properties of hatching might still have significant effect determining the hatching rate. A limitation of the current study is the missing standardisation, and physiological vitality assessment of the sampled embryo pools at t55. The inlet jets of the incubator chambers produce a circular revolving current keeping the developing embryo in motion (Mordenti et al., 2014). Possibly, even if the embryo has reduced, or missing buoyancy, which could potentially also explain the wide ranges of *EHE* mRNA content and collagenase-like activity in the t55 groups. It is not an everyday experience to study the developmental aspects of this critically endangered species. Its complexity render any rearing activity an even more complex task. The great variability in reproductive performance remains an important limiting factor for sampling, while handling and completing its reproduction to close the life cycle, a great challenge for future research.

In conclusion, while the variable hatching rates do not seem to be due to lacking expression or activity of EHE for the chemical digestion of the ZR, the physical and dynamic aspects of hatching, such as the thickness of the chorion layers, and the embryonal movement can potentially vary between maturation inducing regimes and be a determining factor impacting the hatching rate. Transcriptome product of eel hatching enzyme is present in embryos before the currently known activation of the zygotic genome, suggesting maternal derivation, or an expression prior to MZT feature similarly to the sea urchin hatching enzyme. Next generation sequencing techniques on different developmental time-points could provide more direct evidence regarding the timing of zygote genome activation, and to further investigate the DNA methylation and chromatin structure mediated regulation of the hatching enzyme expression (Ferg et al., 2007; Potok et al., 2013; Skvortsova et al., 2019). Considering also the found correlations and the preliminary insights of intracellular Zn^{2+} concentrations, these results are further emphasizing on the importance of nutritional status of female eels when selecting high-quality broodstock for aquaculture and repopulation purposes.

CRediT authorship contribution statement

Bálint Lóránt Hausz: Writing – original draft, Methodology, Formal analysis. **Antonio Casalini:** Writing – original draft, Methodology, Formal analysis. **Domenico Ventrella:** Writing – review & editing, Validation, Data curation, Conceptualization. **Martina Bertocchi:** Writing – review & editing, Methodology, Formal analysis. **Augusta Zannoni:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Nadia Govoni:** Writing – review & editing, Methodology, Formal analysis. **Laura Gentile:** Writing – review & editing, Formal analysis. **Annalisa Zaccaroni:** Writing – review & editing, Methodology, Formal analysis. **Oliviero Mordenti:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Albamaria Parmeggiani:** Writing – review & editing, Supervision, Resources. **Maria Laura Bacci:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Alberto Elmi:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation.

Ethics approval

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (DIR. 2010/63/UE) and the regulations of the Ethics Committee of Bologna University (ID 1157).

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Oliviero Mordenti reports financial support was provided by LIFE Programme. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2026.744194.

Data availability

The datasets generated and/or analysed during the current study are available on the UNIBO Institutional Repository AMSActa, at doi https://doi.org/10.6092/unibo/amsacta/8550.

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