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## **Initial ontogeny of digestive enzymes in the early life stages of captive-bred European eels during fasting: a partial characterization**

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### **Abstract**

The European eel has recently been included on the Red List of the International Union for Conservation of Nature (IUCN) as a critically endangered species. The rearing of *Anguilla* larvae is seen as a key bottleneck to the mass production of glass eels since very little ecological information is available regarding their natural nutrition. Studies of digestive physiology and ontogenetic development in eel larvae could provide useful information for solving some of the puzzles regarding larval fish culture. The aim of this study was to characterize the ontogeny of pancreatic enzymes (trypsin, lipase and amylase) and a peptide hormone regulator of pancreatic secretion (cholecystokinin) in terms of gene expression in European eel larvae from day 0 (P0) of hatching to 5, 10, 15 and 20 days post hatching during fasting. The results in the present study showed that all the genes selected were present, with different levels of expression and increasing trends, during larval development. At P0, the increase in the gene expression of lipase and amylase was higher than that of trypsin and cholecystokinin, confirming that enzymatic activity began before mouth opening and that larvae, provided with a complete enzymatic set, might have the capacity of digesting and absorbing various nutrients.

Keywords: European eel; larval culture; digestive enzyme; gene expression

The European eel (*Anguilla anguilla*) has recently been included on the Red List of the International Union for Conservation of Nature (IUCN) (IUCN, 2014) as a critically endangered species. This has led to the imposition of a short fishing season, a minimum capture size, the protection of larvae and trade regulations to protect this species. One way to protect the species, and to improve its survival and the economy related to it, would be to produce glass eels via artificial reproduction. This would stop the fishing pressure on the wild stocks. To date, larval survival and weaning remain the most problematic aspects hindering the mass production of glass eels.

The development of suitable protocols for larval nutrition is difficult given the lack of relevant ecological information.

Studies regarding the ontogenetic development and the digestive physiology of eel larvae could provide useful information for the larval fish culture and could help in finding solutions to nutritional problems. Partial characterization of the pancreatic digestive enzymes was carried out for the first time in *Anguilla japonica* larvae (Kurokawa et al., 2002; Murashita et al., 2013) and, recently, also in European eel larvae (Politis et al., 2018).

The present study focused on the gene expression of the main pancreatic enzymes trypsin a and b (*try-a* and *try-b*), lipase (*lip*) and amylase (*amy*), and cholecystokinin (*cck*), an enteric hormone regulator of

pancreatic secretion, in captive bred larvae obtained from a population of wild *A. anguilla* from the North Adriatic Sea.

The European eel larvae were produced by artificially induced spawning at the laboratory of the Dept. of Veterinary Medical Sciences of the University of Bologna, Italy using a previously described protocol (Mordenti et al., 2018). The wild eels were caught in a brackish water lagoon; 3 female eels (*AA-001*, *AA-002* and *AA-003*) with *Silver Index V* and 20 wild silver males were selected for reproduction (Mordenti et al., 2013). One thousand five hundred female larvae were randomly divided among three tanks designed for the maintenance of planktonic animals at a density of 31.25 larvae/L and reared until 20 days post-hatching (dph). The larvae were not fed for the entire 20-day period, and were maintained in seawater (salinity  $\cong 30\text{‰}$ ) at a temperature of  $20\pm 0.5^{\circ}\text{C}$ . At 0 (P0), 5 (P5), 10 (P10), 15 (P15) and 20 (P20) dph, five larvae/tank were sampled and immediately placed in 0.5 ml of TRI Reagent (Molecular Research Center, Inc.) and frozen at  $-80^{\circ}\text{C}$  until RNA extraction.

The survival rate was estimated at 5, 10, 15 dph using a volumetric system (number of larvae in 3 one-L samples) and at 20 dph by counting all the live larvae in each tank. The RNA was extracted from three female samples (five larvae/sample) from P0 to P15 (9 samples for each experimental point) while, at P20, the RNA was extracted from only two females (n=5 samples).

For RNA extraction, the samples were lysed after the addition of 2  $\mu\text{l}$  of Polyacryl Carrier TM (Molecular Research Center, Inc, cat. no. PC 152) for 30 sec using a TH Tissue Homogenizer (Omni International) and were then processed as previously described (Tubon et al., 2019). Total RNA (500 ng) was reverse-transcribed and quantitative real-time polymerase chain reaction (PCR) (qPCR) was carried out, as previously described (Parmeggiani et al., 2015) for target genes (*amy*, *lip*, *cck*, *try-a*, *try-b*) and for the reference gene beta-actin ( $\beta$ -*act*). The primer sequences, expected PCR product lengths, accession numbers and melting temperature are reported in Table 1. Real time efficiency was evaluated for all the genes using amplification (each sample in triplicate, 10  $\mu\text{l}$ /well) of a standardized amount of pooled cDNA derived from the liver and the intestine of *A. Anguilla*, starting with 150 ng with 5 subsequent dilutions (75, 15, 3, 0.6 and 0.12 ng). The calibration curve data (efficiency, slope and R<sup>2</sup>) are shown in Table 1. The specificity of the PCR products amplified was confirmed by agarose gel electrophoresis and melting curve analysis.

Delta Ct was calculated as  $\text{Ct}_{\text{target gene}} - \text{Ct}_{\beta\text{-actin}}$ ; the  $2^{-\Delta\Delta\text{Ct}}$  method was then used to analyze the relative expression as fold change (Livak and Schmittgen, 2001) calculated relative to the P0 group.

The mRNA data were first tested for normality using the Shapiro-Wilk test and were then analyzed using one-way analysis of variance (ANOVA) and the post hoc Tukey test (Graph Pad Prism 5 version 5.03; GraphPad Software, Inc., San Diego, CA). Differences were considered significant at  $P < 0.05$ . The survival rate was analyzed using ANOVA and the post hoc Tukey test.

All the eels were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir. 86/609/EEC) and the Ethics Committee of Bologna University regulations (prot. 19/6912).

A statistically significant reduction in larval survival was observed from P5 to P20 (Fig. 1A) when survival was 0.9%, 1.2% and 0% for *AA-001*, *AA-002* and *AA-003*, respectively. The larvae from *AA-003* did not survive beyond P15, coinciding with resorption of the yolk sac. In the present study, the Authors produced the offspring of European eels which were successfully maintained during the yolk sac phase

until the first feeding stage with the aim of documenting the initial ontogeny of the digestive enzymes just before and just after the onset of natural feeding. Contrary to *A. japonica*, in which the life cycle is closed (Murashita et al., 2013), in *A. Anguilla*, research is frozen at the identification of the first food eaten by the larvae.

The larval morphological development observed during the first two weeks post hatching was similar to that reported by other authors (Politis et al., 2017; Sørensen et al., 2016) in European eel larvae maintained at the same temperature (20°C), i.e. the start of mouth opening occurs at 3 dph, the development of the upper and lower jaws at 8 dph, and complete development of the feeding apparatus (with developing teeth becoming increasingly visible and protruding) at 10–13 dph. Subsequently, when all the yolk materials are depleted, any morphological comparisons with other studies are more difficult since all morphological changes depend on yolk sac depletion. In fact, without any exogenous feeding, larval development in this phase depends on two variables: the initial size of the yolk sac which differs among females (Sørensen et al., 2016) and the speed of depletion of the yolk which depends on different larval rearing methods (water temperature and flow rate, different types of tanks, etc). These two factors might explain the differences in maximum survival during fasting obtained in the present trial (20 dph) with respect to what has been reported by Sørensen and colleagues (25 dph).

The qPCR detected the expression of target genes in all the European eel larvae pools from P0 to P20 (Fig. 1B, C). The expression of genes encoding lipase and amylase increased during early development (P5); subsequently, the amylase expression level remained constant until P20 while that of lipase increased to reach a maximum at P20 (approximately a 1390-fold of increase, Fig 1C). During larval development, *Try-a* and *Try-b* showed a significant decrease between P0 and P5 after which the expression of *Try-a* progressively increased until P15/20 while *Try-b* remained similar to P0. Similarly, *ckk* expression decreased until P5, but then increased slightly at P10/15 (Fig. 1B).

The data in the present study showed the presence of the transcript genes in eel larvae from hatching to P20 in accordance with other studies reporting on the European eel (Politis et al., 2018), *Anguilla japonica* (Kurokawa et al., 2002; Murashita et al., 2013; Pedersen, 2003) and other marine species, such as totoaba, gilthead seabream, Pacific bluefin tuna, Asian seabass, Atlantic cod, European seabass and Senegal sole (Galaviz et al., 2015; Mata-Sotres et al., 2016; Murashita et al., 2014; Srichanun et al., 2013; Zambonino-Infante et al., 2008).

The significant increases in amylase and lipase expression are partially in agreement with other results in the European eel (Politis et al., 2018), the gene expression being upregulated in both experimental models but at different times. It is important to note that the present investigation started at day 0 of hatching (P0) unlike that of the study of Politis which started later (4 dph); therefore, the present study was able to identify an earlier variation in gene expression. In the present study, an early increase (P5) in amylase and lipase was observed while, in the study of Politis, a significant increase started later (P15 or P12 post hatching for amylase and lipase, respectively). The differences in gene expression observed may reflect the different parental origins and/or different models of larval rearing.

In the present experimental model, cholecystokinin and trypsin-a expression showed a decrease at P5; cholecystokinin then reached its maximum value at P10 while trypsin-a showed an increasing trend until P20. The increase in trypsin-a level was related to a higher level of cholecystokinin transcripts from 10

dph which seemed to indicate that this gut hormone could play a role in controlling proteolytic digestive activity in eel larvae.

The data in the present study indicated that, when larvae move from endogenous to exogenous feeding, they have a complete enzymatic set and, thus, their capacity of digesting and absorbing various nutrients is potentially good. Moreover, taken together, the present results showed that an increase in the transcript level of lipase was higher than those of amylase and proteases. These results allow hypothesizing that a diet with a higher lipid content is more suitable for feeding the larvae immediately after the resorption of the yolk sac in accordance with a Japanese study (Okamura et al., 2019; Yamada et al., 2019) which used shark eggs, characterized by a higher lipid (22-30%) and a lower protein (19-25%) content (Remme et al., 2005). In fact, while *A. japonica* research, has already addressed the physical characteristics of the diet (Okamura et al., 2019; Yamada et al., 2019), as far as the European eel is concerned, additional investigation regarding digestive physiology and enzymatic activity is still needed.

In conclusion, this study confirmed the possibility of investigating the digestive physiology and nutritional requirements of European eel larvae, in the experimental set of artificially produced offspring in this study.

#### **Conflict of interest**

We declare that there are no conflicts of interest in the authorship or publication of this paper.

#### **Acknowledgements**

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**Table 1** Forward and reverse primer sequences, polymerase chain reaction (PCR) product lengths, melting temperature (T<sub>m</sub>), accession number (AN) in the NCBI (National Center of Biotechnology Information) database and qPCR data of the calibration curve for each gene (Efficiency %, Slope and R<sup>2</sup>).

#### **Figure legend:**

Fig. 1: Survival rate (% , mean ± SD) of European eel larvae evaluated from day 0 of hatching (P0) to 5 (P5, 73,63%±8,22), 10 (P10,53,40%±13.99), 15 (P15, 36,99%±19,13) and 20 (P20, 1,067%±0,3) days post hatching. Different letters indicate statistically significant differences among the groups (P < 0.05, one-way analysis of variance (ANOVA) followed by the post hoc Tukey test) (A). The gene expression pattern of amylase, lipase, cholecystokinin, trypsin-a- and trypsin-b in the European eel larvae analyzed at different experimental times: day 0 (day of hatching) (P0) and 5, 10, 15, 20 days post hatching (P5–P20). The relative gene expression was evaluated as fold of change in relation to the P0 group using the 2<sup>-ΔΔCT</sup> method. Error bars represent the range of expression. (Different letters indicate statistically significant differences among the groups for each gene studied (P < 0.05, one-way ANOVA followed by the post hoc Tukey test) (B). Data regarding the relative mRNA expression (RE) and the range of expression (upper or lower range, UR or LR respectively) (C).

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**Table 1.** Forward and reverse primer sequences, PCR product lengths, melting temperature (T<sub>m</sub>), accession number (AN) in the NCBI (National Center of Biotechnology Information) database and qPCR data of calibration curve for each genes (Efficiency %, Slope and R<sup>2</sup>).

Gen	Sequence (5'-3')	PCR length	T <sub>m</sub> (°C)	AN	Reference	Eff (%)	Slope	R <sup>2</sup>
<i>amy</i>	For.:	90	83	AB07072	(Murashita et	-		
		CGTCATATCGTGGATGGA		1	al., 2013)	88.	3.63	0.99
		AAGGACCAG				3	8	0
	Rev.:	CGGGTTGATGAGGACAGG						
	CTTGGTA							
<i>lyp</i>	For.:	125	85	AY37218	(Murashita et	93.	-	0.98
		GCTCCTCCTGACTGGGACAATGAGT		2	al., 2013)	5	3.48	0

	Rev.:					8		
	TCTCGGTGTCGATGTAGGCTTCGTA							
<i>cck</i>	For.:	120	83.	AB10955	Present study	92.	-	0.97
	AGAAGATTC AAGATTC AACCTCCAGT		5	6		6	3.51	8
	G						3	
	Rev.:							
	CCTGCTCTCATCCTGTGTGTTTG							
<i>try-a</i>	For.: ATCACCTCCACCATGTTCTG	132	87	AB07072	Present study	84.	-	0.97
	Rev.: CTCCGCACAACCGTATCC			0		0	3.77	1
							6	
<i>try-b</i>	For.: CCTGGTCAACGAGAACTGG	141	87.	AB51469	Present study	87.	-	0.95
	Rev.: TTGCGGAGAACCTTGGAAG		5	3		8	3.65	9
							3	
$\beta$ - <i>act</i>	For.: AGCCTTCCTCCTGGGTATG	101	84	DQ28683	(Parmeggia	99.	-	0.99
	Rev.: GTTGGCGTACAGGTCCTTAC			6	ni et al.,	6	3.32	5
					2015)		9	

### Highlights

Gene expression of lipase, amylase, trypsin and cholecystokinin in *A. anguilla* larvae was studied  
 All the transcripts were present with different level of expression during 0-20 dph interval  
 A higher increase of lipase expression than those of amylase and proteases was evidenced

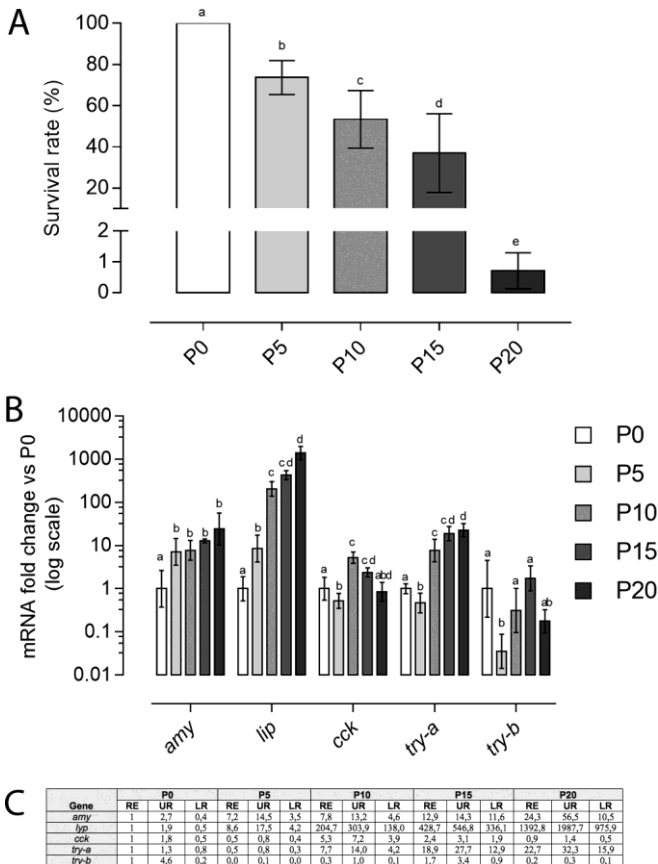


Figure 1