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Maturation of the digestive system of Downs herring larvae (*Clupea harengus*, Linnaeus, 1758): identification of critical periods through ontogeny

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Abstract :

Digestive system functionality is a key process linked to larval recruitment and survival. However, little is known about organ development and enzyme maturation of the digestive system of North Sea Atlantic herring (Clupea harengus). In this study, herring larvae were reared at 13 °C from hatching to 69 day post hatch, covering four developmental stages: (1) yolk sac (8–9 mm), (2) pre-flexion (9–14 mm), (3) flexion (12–18 mm) and (4) post-flexion stages (15–30 mm). Combined histological (semi-quantitative scoring) and enzyme analyses (pancreatic and intestinal) showed that developmental stages are strongly linked to physiological changes. The larvae lack a functional stomach and use the intestine as the primary site of digestion which is mainly supported by pancreatic enzyme activity. The intestine acquired adult enzymatic digestive features with a functional brush border at the end of the flexion stage and pyloric ceca started to develop during the post-flexion stage. The transition from pre-flexion to flexion stage and the end of the post-flexion stage are energetically taxing periods as indicated by a reduced number and size of liver vacuoles. Based on these findings, we consider these moments as critical periods, where herring larvae could be dramatically affected by suboptimal feeding conditions in the field. This implies that pre-flexion stage larvae with low or no liver reserves may not be able to proceed to the next developmental stage. Hence, the level of energy storage in first-feeding larvae needs to be examined for its use as a field indicator of survival and development.

1. Introduction

North Sea herring is a commercially and ecologically important stock of North Atlantic herring (*Clupea harengus*). This stock has experienced high biomass fluctuations, with an almost complete fisheries induced collapse in the past, resulting in the closure of the fishery in the 1980's (Dickey-Collas et al. 2010). Since then, the stock has recovered and, as a result of the very tight regulations, is currently sustainably exploited. Nevertheless, years of poor recruitment, i. e. the quantity of juvenile fish entering the adult population, are regularly reported despite high adult biomass levels. This suggests that other poorly understood factors are responsible for variability in larval survival (Payne et al. 2009)**.**

The economic implications of fish stock inter-annual variability have motivated research on population dynamics and fish biology for more than a century (Hart and Reynolds 2002). Hjort (1914) was the first to point out larval survival, and not only spawning stock biomass, as a key factor determining annual recruitment and size of fish populations. He defined the transition from endogenous to exogenous nutrition as the critical period ("Critical period hypothesis"), i.e. the period when the larvae start feeding, and are most sensitive to starvation. This first-feeding stage has been shown to be a bottleneck period for Western Baltic Sea herring, where young larvae reaching this critical period before the plankton bloom contribute little to the surviving year-class (Polte et al. 2014). Different recruitment hypotheses based on hydrodynamic and trophodynamic factors have been put forward since, and it is now well accepted that biological and physical processes act in concert to determine the survival of fish at early life stages (Houde 2008), from fertilization to metamorphosis and beyond. For instance, feeding success and subsequent growth rate are advocated as two of the most important factors influencing larval survival and recruitment (Anderson 1988; Bergenius et al. 2002; Jenkins and King 2006; Robert et al. 2007).

Thus far, condition and physiological state of field-caught larvae have been used as indicators of suitable environmental condition and potential recruitment success. These are typically measured by indices, including morphometric, histological and biochemical measurements (Ferron and Leggett 1994). These indices are used to assess directly or indirectly the nutritional status of organisms, or to infer their ability to bear starvation (Ehrlich et al. 1976). Some of them are widely used in larval fish surveys, such as the relative content of RNA to DNA in tissue, or the size corrected weight of individuals. Recently, Denis et al. (2017) observed that Downs herring larvae, one of the most important spawning components of North Sea herring, undergo a change in their condition at around 13 mm. The authors observed a reduction in instantaneous growth rate (by RNA: DNA) along with an increase in ingestion rate and suggested this to be a possible critical period resulting from a change in

the energy-allocation strategy (i.e., from growth to energy reserves). Because the response of RNA:DNA ratios differs with larval size (Foley et al. 2016) the former hypothesis of a second critical period needs to be validated with additional independent and well-controlled approaches. It is known that ontogeny and organogenesis of the digestive system can influence the physiological status of larvae (Suthers 2000). Hence, knowledge of the development and maturation of the digestive tract, as well as the dynamics of energy storage by accessory glands, would be very helpful for a precise interpretation of field data. Furthermore, the study of ontogenic transitions could be of importance to determine critical periods during the development at which suboptimal feeding conditions could directly affect survival and recruitment.

Marine fish larvae are not fully developed at hatch and undergo simultaneous major ontogenic changes in morphology, anatomy and physiology. Growth and development are linked to the nutritional status of individuals which relies on internal storage, and after first feeding, on food quality, availability and functionality of digestive organs (Ronnestad et al. 2013). Maturation of the larval digestive tract is defined as the acquisition of adult digestive features; this process has been widely studied for digestive enzymes in teleosts, especially in the context of aquaculture (Zambonino Infante and Cahu 2001). Most fish species lack a morphologically distinct and functional stomach (characterized by the appearance of gastric glands, (Zambonino Infante and Cahu 2001)) before metamorphosis, and digestion processes in larvae mainly depend on other digestive organs, especially the pancreas (Ronnestad et al. 2013). Among pancreatic enzymes, trypsin activity has been related to food ingestion (Pedersen et al. 1987) and has been used to experimentally identify starving from well-fed herring larvae. Trypsin measurements reflect changes in food availability and nutritional condition over shorter timescales than RNA/DNA ratios which has been estimated > 5 days (Ueberschär and Clemmesen 1992; Foley et al. 2016). Intestine maturation is characterized by changes in enterocytes enzymatic activities, i.e. a decrease in cytosolic activities concomitant with a sharp increase in brush border membrane enzyme activities (Zambonino Infante et al. 2008). Consequently, digestive enzymes activities are used as indicators of larval development and potential of survival. Although these aspects are essential to understand larval recruitment, which is necessary to assess stock fluctuations, there is a striking lack of information on the ecologically and economically important North Sea *C. harengus*. Teleosts have similar developmental patterns, but large species-specific variations exist regarding organ differentiation timing and metabolic efficiency (Gisbert and Sarasquete 2008). For a more complete assessment of potential recruitment success it is hence essential to acquire in-depth knowledge on the processes during larval development, both structural changes as well as changes in physiology.

In the present study, we focused on North Sea herring (Downs component) and aimed to (1) describe the development and maturation of the digestive tract and associated organs (oesophagus, stomach, intestine, liver and pancreas), and (2) identify physiological changes that could potentially be linked to critical periods during ontogeny. To do so, herring larvae were reared in the laboratory from hatch to the last larval stage, and digestive enzyme activities (trypsin, alpha amylase, leucine-alanine peptidase, aminopeptidase N and alkaline phosphatase) as well as histological structure of digestive organs were assessed at different times through development. Hence, the present study provides important basic information on the structural and biochemical changes in the digestive system throughout the larval stages of herring that can be useful for future work assessing the condition of field-caught larvae in relation to environmental conditions.

2. Materials and methods

2.1 Artificial fertilization

Mature herring from the Downs component were obtained in November 2018 from a local organization of fishermen (Coopérative Maritime Etaploise, CME) in Boulogne-Sur-Mer (France). Artificial fertilization was carried out using ready-to-spawn fish at the Ifremer Manche-Mer du Nord laboratory. Eggs of 27 females (mean standard length: 27.2 $\pm$ (SD) 1.92 cm; mean weight: 166.4 $\pm$ 32.54 g) were stripped-spawned in seawater and placed on four plastic plates (PVC) of 490 cm^2 each before being fertilized with the sperm of 9 males (mean standard length: 28.0 $\pm$ 3.84 cm; mean weight: 179.2 $\pm$ 32.0 g). The eggs and sperm were incubated for 10 minutes at 13.5°C and 32.7 psu, before rinsing. To ensure genetic diversity, on each plate eggs from at least six females were spread and the mixed sperm of at least two males was added for the fertilization. Along with the four PVC plates a smaller slide of 21 cm^2 was used to monitor closely the fertilization and eggs development without having to take out of water the eggs intended for the experiment. Eggs were stripped-spawned on the slide and fertilized with the same protocol in order to give us an approximation on fertilization success. The fertilization success of the slide was of 42%. Around 200 eggs were laid on 21 cm^2, thus we can estimate that approximately 4600 eggs were present on each plate of the experiment.

The plates were transported to Ifremer-Centre de Bretagne laboratory in boxes with oxygenated and thermoregulated sea water (variation range: 13.5 to 15°C). Eggs were then incubated in a 200 L tank in the dark. For the entire experiment, natural seawater was pumped from the Bay of Brest (32.5 psu, pH 8.0), passed through a sand filter, heated to avoid natural temperature fluctuations, filtered (2 µm) and UV sterilized (PZ50, 75W, Ocene, France).

2.2 Larval rearing

Most of the eggs hatched on the 9th day of incubation (starting point of the experiment: 1 dph) and larvae were kept until 69 days post hatch (dph). Three days after hatching (3 dph) about 4500 larvae were counted and distributed equally in three 38 litres conical black tanks (1500 larvae by tank), constituting the replicates of the experiment. A continuous flow-through system of 20 L h^{-1} was used and the temperature was set to 13°C (13.3 ± 0.25°C). Oxygen saturation was measured daily with an oximeter (WTW Oxi 340, Bioblock scientific) and was always higher than 98%. In order to prevent any food limitation, the daily food quantity was distributed four times during the day to maintain an *ad libitum* level, ensuring that there were always prey in the tank during the day. To be sure to allow proper feeding through time we used an increasing range of living prey sizes from phytoplankton to 24 h old nauplii, before weaning with feed granules.

Rhodomonas salina (Strain 2002) and *Oxyrrhis marina* (Strain 21.89) were obtained from the Culture Collection of algae (University of Göttingen, Germany). *R. salina* were grown in 10 L bottles under constant light in enriched Conway medium to provide quick growth (N and P concentrations doubled). *O. marina* were cultivated in a 5 L bottle under constant lighting and fed *R. salina*. Nauplii of the commercially available *Artemia salina* (VNBS, Viepearl) were hatched in 60 L tanks after 24 h at 28°C, and directly used to feed larvae early in the development. Thereafter, artemia nauplii were enriched 24 h (A1) in a mixture of fish oil (FO) and baker yeast (BY) (For 1 Million artemii = 4.3 g FO and 11.1 g BY).

The feeding protocol of Moyano et al. (2016) was followed for early feeding. Yolk sac larvae were fed the microalgae *R. salina* (mean: 30 000 cells mL^{-1}) and the heterotrophic protist *Oxyrrhis marina* (mean: 250 cells mL^{-1}). Two days after mouth opening, *Artemia salina* nauplii (A0) were supplied in the tanks (mean: 70 A0 larvae^{-1} d^{-1}). From 22 to 39 dph a mix of *A. salina* nauplii (mean: 140 A0 larvae^{-1} d^{-1}) and 24 h old enriched artemii (mean: 140 A1 larvae^{-1} d^{-1}) were added. After that, A1 were supplied in decreasing quantities (mean: 200 A1 larvae^{-1} d^{-1}) while introducing feed granules (Neo Supra, Le Gouessant, 58% proteins, 13% lipids) until 49 dph (from 0.2 to 0.5 g tank^{-1}d^{-1}). From 49 to 69 dph, larvae were fed only with the granules (1 g tank^{-1} d^{-1}). The lighting sequence of the tanks was modeled on aquaculture protocols. The lighting period was of 24 h a day, progressively increased from 1 to 59 lux along the development, in order to maximize larval feeding, in particular for young herring larvae which possess only cone cells prior to metamorphosis (Bell and Dick 1993).

2.3 Larval sampling

A total of 761 larvae of *C. harengus* were sampled and measured (Total Length, TL in mm) throughout the experiment to estimate growth rates. A subsample of those individuals were used for

enzyme analysis. Larvae were collected with a sieve in the morning, before feeding, and euthanized in ice water. As larvae are fragile organisms, homogeneous sampling through the tanks inevitably leads to a slight increase in mortality. Thus, for histological individual analysis we decided to limit the number of larvae sampled at the beginning of development and to increase it progressively according to the progress and success of the rearing. The samplings were adjusted to not go below a minimum, non-stressful, density of 25-30 larvae.L^{-1} per tank. Four vitelline larvae were directly sampled from the hatching tank at 3 dph. After that, from 2 to 13 larvae per tank were sampled at 12, 19, 21, 25, 28, 35, 49 and 69 dph. The larvae were preserved for 48 h in Bouin's solution, then rinsed and preserved in 70% ethanol in the dark until analysis. To describe the morphological development, larval stage and size were defined under binocular observations for 4 larvae (at 3 dph) + ((2-10 larvae) * 7 (sampling dates) * 3 (replicates)) + (13 larvae * 3 (replicates)) = 169 larvae. To have a reference value on the level of shrinkage of the conservation technique, larvae sampled at 69 dph were measure before and after conditioning (13 larvae per tank). A total of 35 larvae were used to histologically describe the digestive system development through time.

The developmental stage (Fig. 1) for each larva was determined according to Doyle (1977): 1) yolk sac larval stage, 2) pre-flexion stage; yolk sac absent and straight notochord, 3) flexion of the notochord stage and 4) post flexion stage with visible pelvic fins.

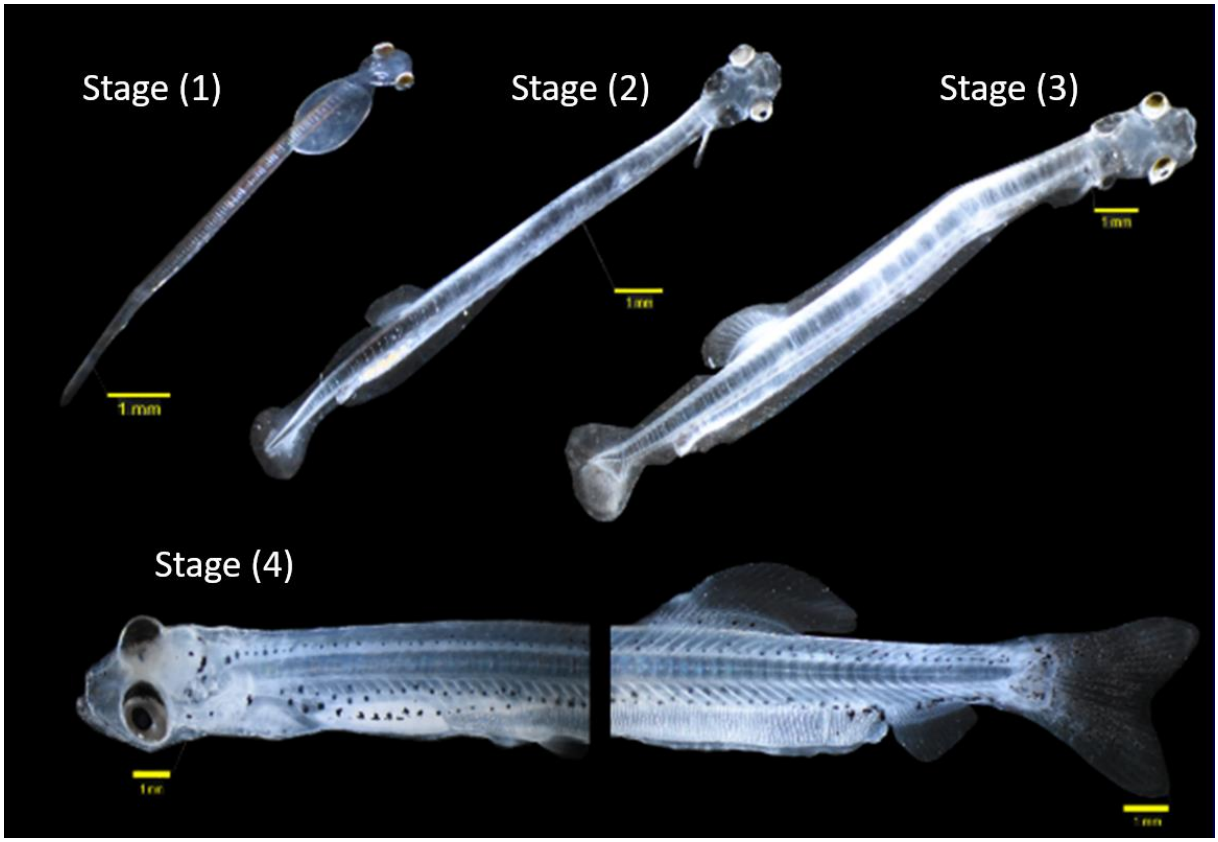


Fig. 1 Developmental stages of herring larvae: (1) yolk sac stage – 3 dph, 8-9 mm TL , (2) pre-flexion stage – 12 dph, 9-14 mm TL, (3) flexion stage – 28 dph, 12-18 mm TL and (4) post-flexion stage, the larvae is cut in two parts because it was unfortunately too long to fit entirely under the binocular – 69 dph, 15-30 mm TL, binocular observations

We sampled larvae for enzyme activity analyses from 14 dph onwards and pooled their tissue for spectrophotometric assays. Between 30 and 50 larvae were sampled in each tank for enzymes analysis at 14, 21, 28, 35 and 49 dph. The larvae were put in vials and stored at -80°C, yielding a total of 5 (sampling days) * 3 (replicates) = 15 samples.

2.4 Organogenesis of the digestive system

Organogenesis of the digestive system was studied using histology. Individuals were dehydrated and embedded in paraffin blocks, as described by Di Pane et al. (2019), substituting the xylene by diasolv (non-Carcinogenic, Mutagenic or Reprotoxic substitute of xylene/toluene). Sagittal section of 7 µm were made with an automated-microtome (Leica, RM2255) and mounted on slides. Larval sections were dewaxed and rehydrated in successive baths of diasolv and alcohol progressively less concentrated (100 %, 95%, 70%) and tap water. Two histochemical colorations were used on different larvae. Half of them were stained using alcian blue (AB) pH 2.5 alone. The other half was double-stained with Periodic acid-Schiff (PAS) to stain glycogen and neutral mucosubstances, and alcian blue (AB) pH 2.5 for acid mucosubstances. Then all the slides were stained with Groat's hematoxylin and picro-indigo carmine for topographic coloration. Between 3 to 6 larvae per sampling date were observed under a microscope (Leica, DM6B) to describe the development of the larval digestive system. We focused on the histological organization of the digestive tract (oesophagus, stomach, intestine) and accessory digestive glands (liver and pancreas) (Table 1). Presence of zymogen, inactive enzymatic precursor, in the pancreas and glycogen, indicative of energy reserve, in the liver were examined. The liver condition and reserve amount were scored based on hepatocytes vacuoles characteristics following the protocol developed by Di Pane et al. (2019). Image acquisition was done with a software (LAS X) and a camera (Leica, DMC4500) connected to the microscope.

Table 1 Synthesis of the histological structures observed for the digestive system development description (Zambonino Infante et al. 2008; Ronnestad et al. 2013; Di Pane et al. 2020)

Organs	Tissue	Features		
Oesophagus	Tube shape	short and narrow	elongated	folded
	Epithelium	simple	pseudo-stratified	stratified

	Epithelial cells shape	cubic		prismatic
	Goblet cells	present		absent
	Mucins	acid	neutral	both
Rudimentary Stomach	Epithelium	simple	pseudo-stratified	stratified
	Epithelial cells shape	cubic		prismatic
	Villi	present		absent
Differentiated Stomach	Cardiac stomach	simple epithelium	prismatic cells	prominent villi
	Fundic stomach	simple epithelium	prismatic cells	gastric glands
	Pyloric stomach	simple epithelium	prismatic cells	pylorus and pyloric caeca
Rudimentary Intestine	Epithelial cells shape	prismatic		
	Goblet cells	present		absent
	Mucins	acid	neutral	both
	Villi	present		absent
	Brush Border	present		absent
Differentiated intestine (anterior and posterior)	Epithelial cells shape	prismatic		
	Goblet cells	present		absent
	Mucins	acid	neutral	both
	Villi	moderate		prominent
	Brush Border	present		absent
	Vacuoles	present		absent
Exocrine Pancreas	Acini stain	dark		pale
	Tissue organisation	Indistinct acini, detachments		Distinct acini, contiguous
	Zymogen	present		absent
Endocrine Pancreas	Langerhans islets	present, number		absent
Liver	**Score**	**1**	**2**	**3**

Hepatocytes size	small	medium	large
Vacuoles	absent	small / medium	large
Glycogen quantity	absent / small	medium	huge (everywhere)
Tissue structure	some detachments	joined cells	joined cells

260

261 **2.5 Enzymatic assays**

262 Activity of five digestive enzymes was assessed during larval development: trypsin (EC 3.4.21.4),
263 alpha amylase (EC 3.2.1.1), leucine-alanine peptidase (Leu-Ala), aminopeptidase N (aminP, EC
264 3.4.11.2) and alkaline phosphatase (AP).

265 Larvae from the first two sampling dates (14 and 21 dph) were entirely homogenized with a polytron
266 (PT – MR2100, Polytron, NR: 324412). Older ones (28, 35, 49 dph) were dissected and measured on
267 ice under a binocular microscope (Cahu and Zambonino Infante 1994) in order to obtain pancreatic
268 and intestinal segments. Samples were homogenized in five volumes (w/v) of ice-cold distilled water.
269 Brush borders were extracted and purified from the intestinal segment (Crane et al. 1979).
270 Homogenates of younger larvae and pancreatic segments from older larvae were used to determine
271 activities of pancreatic enzymes, i.e. trypsin and alpha amylase, according to Holm et al. (1988) and
272 Métais and Bieth (1968) respectively. Cytosolic leucine-alanine peptidase activity was assessed from
273 intestinal homogenates (Nicholson and Kim 1975). Assays of the brush borders enzymes were
274 performed for alkaline phosphatase and aminopeptidase N following Bessey et al. (1946) and
275 Maroux et al. (1973), respectively. All measurements were carried out using a spectrophotometer
276 (Thermo Scientific, Evolution 201) at 37°C.

277 For each analysis, protein content was determined in the sample with Bradford's method (Bradford
278 1976). All the activity measurements were done three times for each sample and averaged to give a
279 value per tank and time. Enzymatic activities were expressed as specific activities in mUnit mg
280 protein^{-1} and segmental activities, i.e. the total activity of one enzyme per larva in the intestinal
281 segment. The level of intestinal maturation was calculated as the ratio of brush borders enzymes
282 related to cytosolic enzymes based on the segmental activities (Zambonino Infante et al. 1997).

283 **2.6 Statistical analyses**

284 Data analyses were performed using the R software (R core Team, 2019) with an alpha level of 0.05.
285 Differences in growth rates among tanks were investigated with a linear mixed-effects model

(LMEM) using the package “lme4” and the function *lmer* (Bates et al. 2015). “Length” was the outcome variable with “dph” used as a fixed effect and “tank” as a random effect. Following recommendation by (Barr et al. 2013) we fit a maximal random effects structure which allows for random slopes (for the different tanks) and random intercepts. The function *ranova()* from the “lmerTest” package (Kuznetsova et al. 2017) was then used to test the effect of the random effect on the model (likelihood ratio tests), and normality of residuals was checked. The random effect “tank” was not significant ($P > 0.05$) and so replicates of the different tanks were pooled for subsequent analysis. The data are expressed as mean $\pm$ SD. Enzymes data normality was checked with Shapiro’s test and homoscedasticity with Bartlett’s test. As normality and homoscedasticity were respected, specific activities and enzymatic ratio data were compared by one-way ANOVA, with time as a factor, followed by post hoc HSD Tukey tests when significant differences were found.

3. Results

3.1 Size distribution during developmental stages

LMEM fixed effect estimate of the variation of length over time was of 0.22 $mm.d^{-1}$ (Table 2).

Table 2 Estimated model fixed effects of the variation of length over time using "tank" as a random effect for 761 observations.

Effect	Estimate	Std. Error	df	t	P value
Intercept	8.87	0.47	2.18	18.59	0.001
Dph	0.22	0.01	2.15	18.10	0.002

All larvae presented a yolk-sac and hatched without a functional mouth but with fully pigmented eyes. The mouth opened at 3 dph (8-9 mm) and the yolk sac was fully resorbed at 8 dph (9-10 mm). Phytoplankton was observed in the intestine from 5 dph (8-10 mm), and artemia nauplii from day 7, before total resorption of the vitellus.

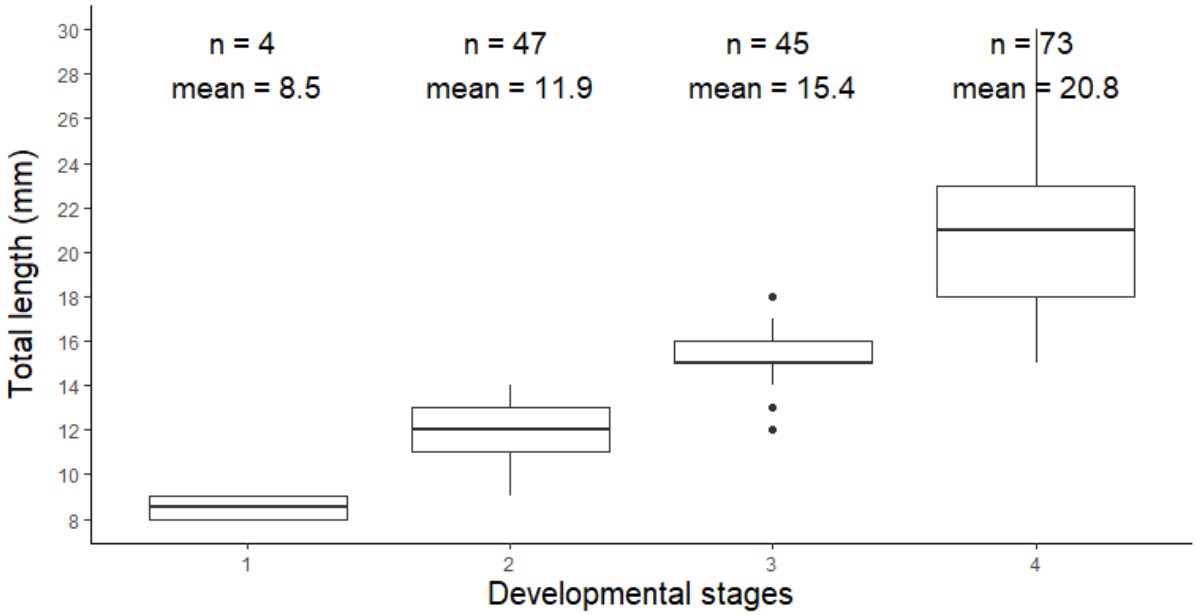


Fig. 2 Boxplot representation of the larval total length (mm) observed for each developmental stage

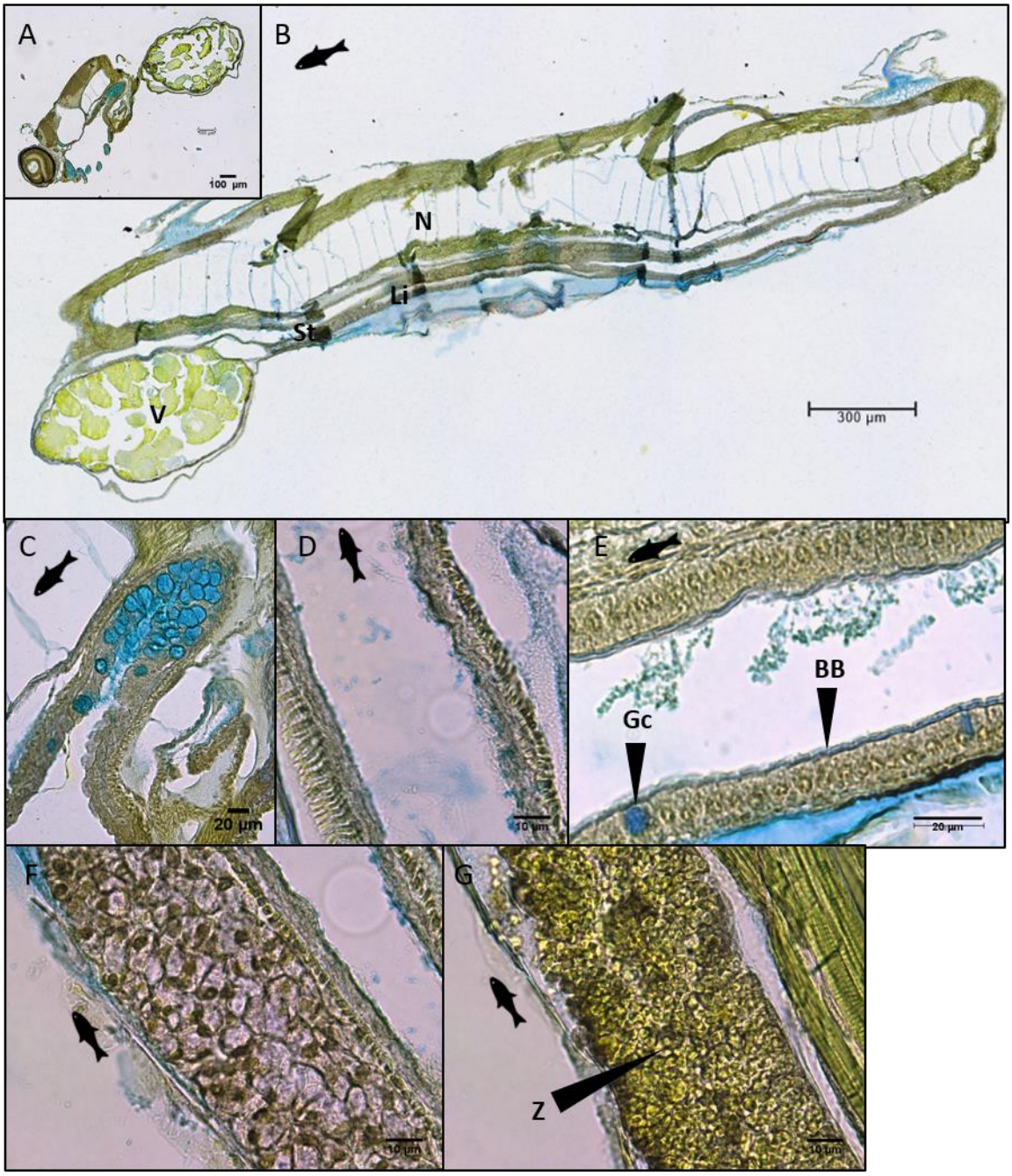
Additional to size measurements, the developmental stage was determined for 169 larvae sampled for histological analysis. Size ranges between each stage are well separated (Fig. 2). Stage 1 larvae were found at 3 dph, the larvae were thin and long with a straight notochord, had a visible intestine and vitelline reserves. In stage 2 larvae between 12 and 35 dph, the vitellus was totally resorbed and the caudal and dorsal fins started to differentiate. For stage 3 larvae between 21 and 35 dph, body height was higher, the intestine bigger and the caudal, dorsal and anal fins fully differentiated. Melanophores were also present along the superior part of the intestine and some above the head and on the caudal fin. For stage 4 larvae from 35 to 69 dph, all the body features were bigger, the whole body was thicker and the pelvic fins started to differentiate. Melanophores were present all over the body and concentrated above the intestine, on the dorsal part of the body, above the head, around the mouth and on the dorsal fin. The measurements came from individuals stored successively in Bouin's solution and 70% ethanol for histology. The conditioning method can result in shrinkage of the larvae. Individuals measured before and after conservation shrank by an average of 11.2 % (± 7.4 %, n = 39).

3.2 Digestive system development

Yolk-sac stage (stage 1)

At 3 dph, larvae were around 8 mm and the mouth was open with the yolk sac still present. The oesophagus was relatively short, narrow and folded, with an important amount of goblet cells (Fig. 3c). Only acid mucosubstances (AB positive, Fig. 4a) were detected. The stomach was not yet

morphologically differentiated and appeared as a simple cavity with short and mostly flattened epithelial cells (Fig. 3d). In the intestine, the enterocytes formed a straight thick layer with some goblet cells in between, producing acid mucosubstances (AB positive). Microvilli forming the brush border at the top of the cells were visible (Fig. 3e). Pancreas and liver had an elongated shape going from the back of the pectoral fins to the beginning of the intestine. Pancreatic cells were organized into weakly distinct clusters with a moderate amount of zymogen (Fig. 3g). Only exocrine pancreas was visible. The liver had large cells with large vacuoles (Fig. 3f) and a pronounced pink (PAS-positive) tint highlighting an important quantity of glycogen in the cells.



340

341 **Fig. 3** Sagittal sections of Downs herring larvae (yolk sac stage, 8 mm, 3 days post hatch), colored
342 with alcian blue pH2.5, Groat's hematoxylin and picro-indigo carmine. **A** Section of the head and yolk
343 sac, Magnification x200, scale bar = 100 µm. **B** General view of the larva, Scan x200, scale bar = 300
344 µm. Magnification x1000; oil immersion for C, D, E, F and G. **C** Details of the oesophagus with goblet
345 cells, scale bar = 20 µm. **D** Section of the stomach cavity, scale bar = 10 µm. **E** Section of the intestine
346 with goblet cells, scale bar = 20 µm. **F** Details of the liver with large vacuoles and glycogen (pink),
347 scale bar = 10 µm. **G** Details of the pancreas with zymogen granules, scale bar = 10 µm.
348 Abbreviations: BB Brush Borders, Gc Goblet cells, I Intestine, Li Liver, N Notochord, St stomach, V

Vitellus, Z Zymogen granules within acinar cells, the black fish icon represents the orientation of the slide to help locate the head and the tail position

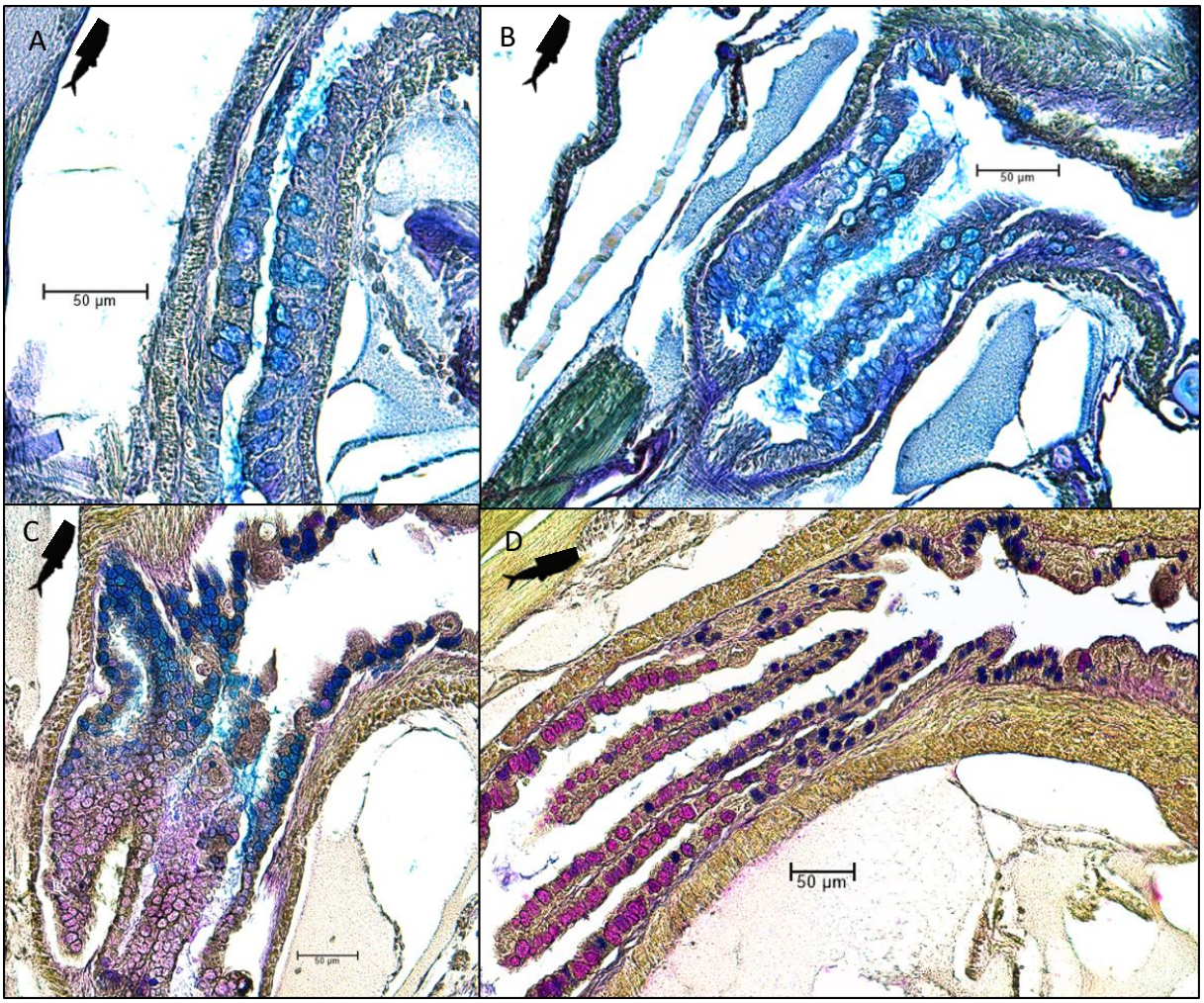


Fig. 4 – Oesophagus sagittal sections of Downs herring larvae at stage 1 (**A**), stage 2 (**B**), stage 3 (**C**) and stage 4 (**D**), colored with periodic acid-Schiff, alcian blue pH2.5, Groat's hematoxylin and picro-indigo carmine. Magnification x200, scale bar = 50 µm, the black fish icon represents the orientation of the slide to help locate the head and the tail position

Pre-flexion stage (Stage 2)

At 12 dph, larvae were around 10 mm, the tissular organization was quite similar to the one described for stage 1 except for the yolk sac which was completely resorbed. The goblet cells in the upper part of the oesophagus were also tinted in blue, revealing acid mucosubstances production (Fig. 4b). Some stage 2 larvae showed few violet goblet cells in the lower part of the oesophagus, revealing both acid and neutral mucosubstances production (violet tint: AB and PAS positive).

Anatomical changes began to appear at 19 and 21 dph when larvae were around 11-12 mm and at the end of the stage 2. The distinction between the stomacal cavity and the intestine was clearly visible as a structured pylorus. In the intestine, two regions could be distinguished: the mid- and the hindgut, separated by a simple constriction of the organ. The epithelial structure was homogeneous for both parts, enterocytes began to form villi and still contained goblet cells. The endocrine pancreas was visible.

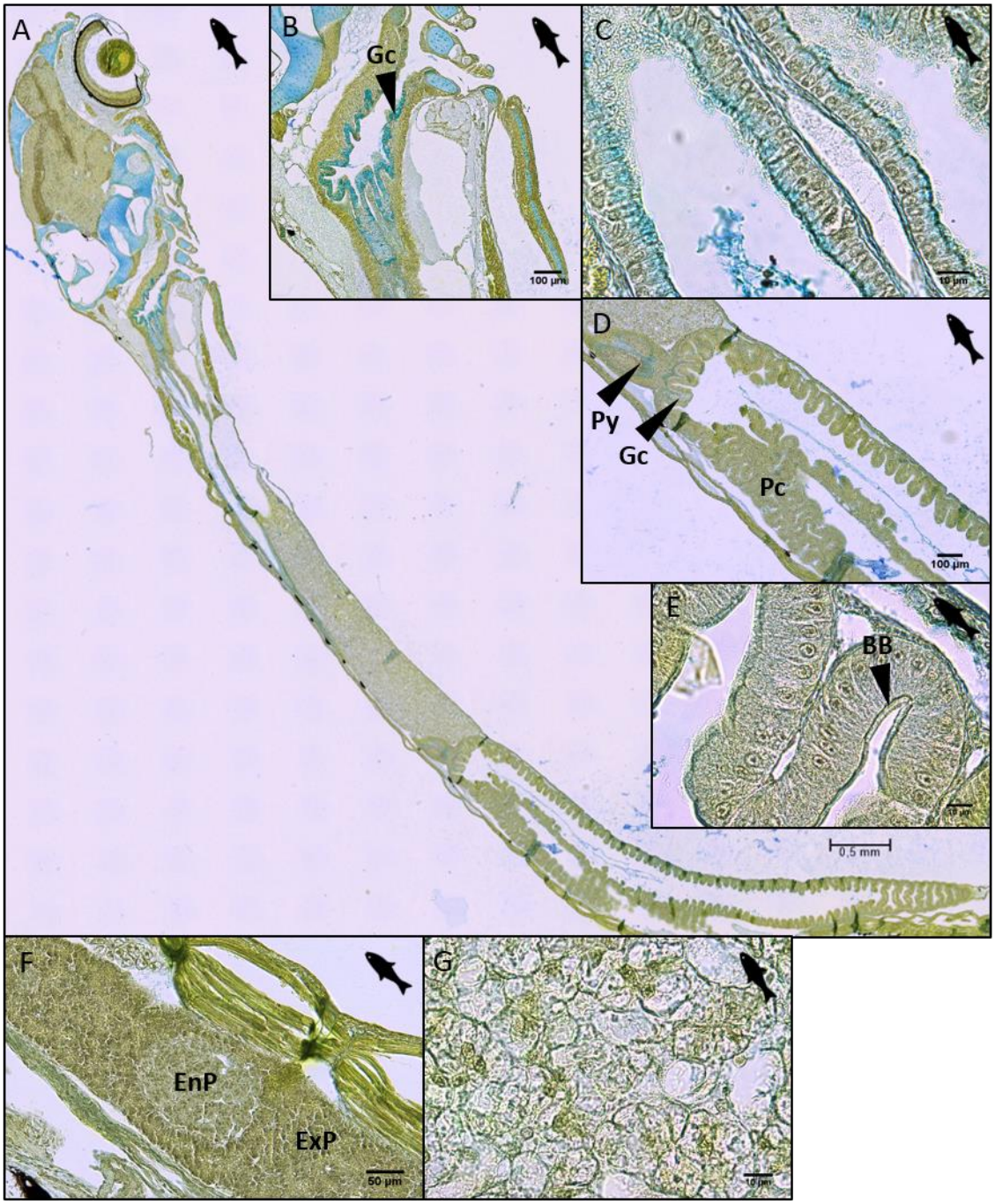
Flexion stage (Stage 3)

At 25 dph, larvae were around 14 mm and started transitioning into stage 3. The global morphology of the digestive tract remained the same but the organs became larger and thicker. The oesophagus interstice was wider and longer. A clear pattern appeared in the mucosubstances secretion by the goblet cells of the oesophagus: the proximal half produced acid mucus and the distal part neutral mucus (Fig. 4c). The oesophagus opened up to a stomacal cavity extended in the upper part. The epithelial cells height of the stomach increased and the villi of the intestine became larger. The endocrine portion of the pancreas took the form of one large cluster of cells called islet, with a central position. Before 28 dph, exocrine pancreatic cells seemed to be regrouped but without a clear pattern. At 28 dph, cells were organized into distinct and circular acini. The pancreas became more elongated, protruding beyond the beginning of the intestine. The liver underwent major changes at the beginning of stage 3, between 25 and 28 dph. Cells were smaller and the vacuoles were reduced or absent for some larvae. At 35 dph (end of the stage 3), the liver showed both large cells with huge vacuoles and small cells with smaller vacuoles. The stain remained pink through the stage highlighting the presence of glycogen reserves.

Post-flexion stage (Stage 4)

Stage 4 is the last stage before metamorphosis into a juvenile. Internal and external morphology of the larvae become increasingly closer to that of adults (Fig. 5a). The oesophagus and its longitudinal folds were longer and the interstice wider than in stage 3 (Fig. 5b). As for the stage 3, two distinct parts were identified by the goblet cells mucus production, the coloration was stronger than before suggesting an increase in the quantity of acid and neutral mucosubstances produced respectively in the superior and inferior part of the organ (Fig. 4d). Epithelial cells of the stomach were higher at 49 dph (Fig. 5c). At 69 dph a coloration appeared in the stomach, the stomacal glands were not observed. Most of the larvae presented a strong blue coloration (AB positive: acid mucosubstances) for two third of the stomach length and a strong pink coloration (PAS positive: neutral mucosubstances) in the last part of the stomach directly connected to the pylorus. At 69 dph, the intestine was larger, the villi were prominent and the ceca pyloric were visible after the pylorus (Fig.

5e,d). At 49 dph the acini were still distinct but at 69 dph the structure of the exocrine pancreas was more diffuse. At the end of the stage 4, one large endocrine islet was still present in the middle of the pancreas and small islets were observed on either side of the larger one (Fig. 5f). The pancreas expanded further along the intestine. The liver of stage 4 larvae, like for stage 3, was highly variable with some individuals presenting numerous and large vacuoles (Fig. 5g, 6a) while for others the vacuoles and amount of glycogen were reduced or absent (Fig. 6b,c).



404

405 **Fig. 5** Sagittal sections of Downs herring larvae (stage 4, 27 mm, 69 days post hatch), colored with
406 alcian blue pH2.5, Groat's hematoxylin and picro-indigo carmine. **A**. General view of the larva, Scan
407 x200, scale bar = 0.5 mm. **B** Section of the oesophagus and upper part of the stomach, Magnification
408 x200, scale bar = 100 µm. **C** Details of the stomach, Magnification x1000; oil immersion, scale bar =
409 10 µm. **D** . Section of the pylorus and start of the intestine with goblet cells, prominent villi and a
410 small part of pyloric ceca, Magnification x200, scale bar = 100 µm. **E** Details of de villus structure of
411 the intestine and the Brush Border, Magnification x1000; oil immersion, scale bar = 10 µm**F** Details of
412 the pancreas organization with pancreatic islets and exocrine pancreas, Magnification x200, scale bar

= 50 µm. **G** Details of the liver with large cells and vacuoles, and a small amount of glycogen (pink), Magnification x1000; oil immersion, scale bar = 10 µm. Abbreviations: BB Brush Borders, Gc Goblet cells, I Intestine, Li Liver, N Notochord, Pancreas with ExP Exocrine Pancreas and EnP Endocrine Pancreas, Oe Oesophagus, Pc Pyloric ceca, Py Pylorus, Sb Swim bladder, St stomach, V Vitellus Z Zymogen granules within acinar cells, the black fish icon represents the orientation of the slide to help locate the head and the tail position

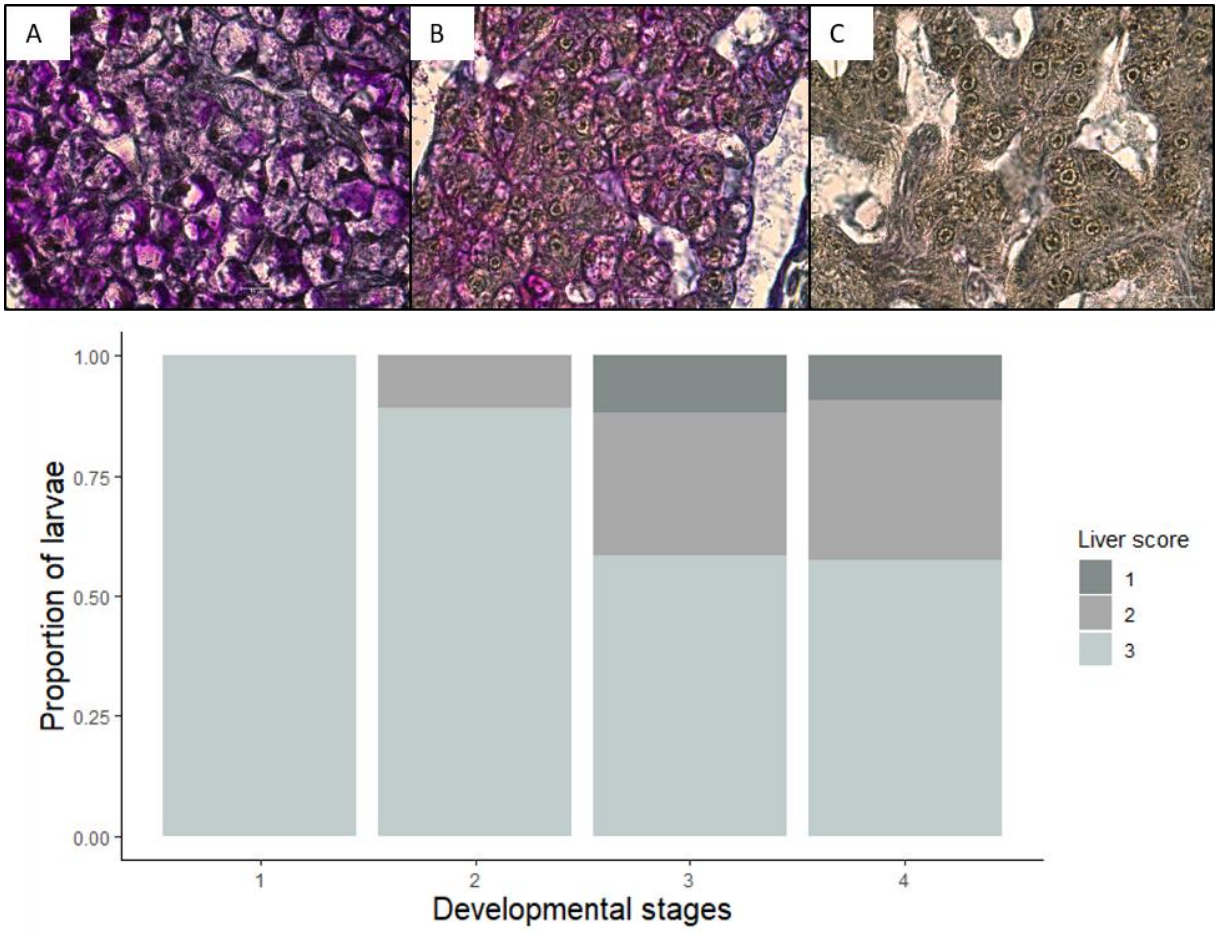


Fig. 6 Sagittal sections of Downs herring liver at different scores and liver score proportion as a function of developmental stages. (**A**) Score 3: wide vacuoles, (**B**) Score 2: scattered vacuoles, (**C**) Score 1: no vacuoles, Magnification x1000; oil immersion, scale bar = 10 µm. (**D**) Larval proportion of liver score for the developmental stages 1 (n = 3), 2 (n = 8), 3 (n = 11) and 4 (n = 13)

3.3 Activity of digestive enzymes

The level of maturation of the digestive system was determined based on the variations in the intestinal enzymes activities (Cahu and Zambonino Infante, 1995). Trypsin specific activity remained constant from 14 to 49 dph (31.0 $\pm$ 5.11 mU mg protein^{-1}; ANOVA, F (4,10) = 1.67, P = 0.233) (Table

3). Amylase specific activity was different through time (ANOVA, F (4,10) = 12.14, P < 0.01), a post hoc Tukey test showed that the activity was higher at 14 dph (P < 0.05) and decreased later from 5079 ± 470.6 to a mean of 818.1 ± 324.7 mU mg protein^{-1} at 49 dph. Intestinal enzymes specific activities were tested for larvae at 28, 35 and 49 dph. No statistical significant differences were found for the intestinal enzymes specific activities Aminopeptidase N (ANOVA, F (2, 6) = 2.79, P = 0.139), Alkaline phosphatase (ANOVA, F (2, 6) = 4.80, P = 0.057) and Leucine-alanine peptidase (ANOVA, F (2, 6) = 0.637, P = 0.561), at the three different ages. The evolution of the ratio between segmental activities of the brush border and cytosolic enzymes differed through time (AP/Leu-Ala: ANOVA, F (2,6) = 9.55, P = 0.014; AminP/Leu-Ala: ANOVA, F (2,6) = 8.37, P = 0.018), a post hoc Tukey test highlighted a shift between 28 and 35-49 dph (Fig. 7, P < 0.05).

Table 3 Summary of the specific activities (mU mg protein^{-1}) of pancreatic (trypsin and amylase) and intestinal (aminopeptidase N, alkaline phosphatase and leucine-alanine peptidase) enzymes during herring larval development. Values represent mean ± SD (n=3), * indicates a statistical difference over time (P < 0.05)

	Enzymes		**Day Post Hatch**				
			14	**21**	**28**	**35**	**49**
	Total length (mm)		/	/	15.0 ± 2.2	16.9 ± 2.0	18.5 ± 2.4
Specific activity (mU mg protein^{-1})	Pancreatic	Trypsin	27.9 ± 6.5	34.9 ± 10.8	29.0 ± 1.1	37.7 ± 6.6	25.5 ± 5.6
		Amylase	5079 ± 470.6*	2215 ± 70.2	1724 ± 1047	1530 ± 1387	818.1 ± 324.7
	Intestinal	Aminopeptidase N	/	/	171.4 ± 17.8	257.8 ± 74.0	215.9 ± 15.3
		Alkaline phosphatase	/	/	164.0 ± 54.5	436.5 ± 166.3	405.8 ± 107.5
		Leucine-alanine peptidase	/	/	395.4 ± 137.2	450.7 ± 87.6	507.0 ± 132.4

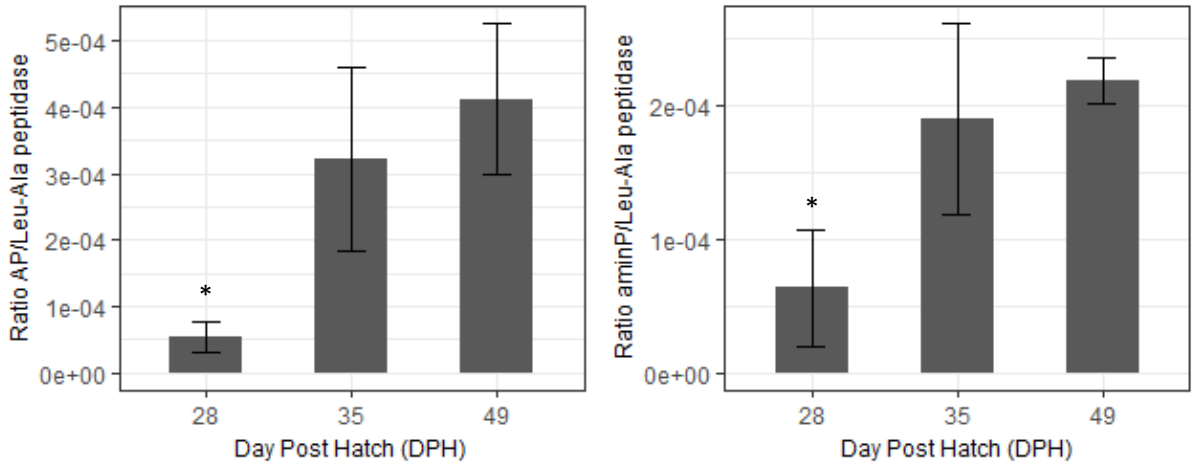


Fig. 7 Intestinal maturation of the enterocytes during *Clupea harengus* larval ontogeny indicated by the change in ratio in segmental activities of brush border enzymes (AP: alkaline phosphatase, aminP: aminopeptidase N) / cytosolic enzymes (Leu-Ala: leucine-alanine peptidase). Results are presented in mean ± SD (n=3). * indicate significant differences between ratios ($P < 0.05$)

4. Discussion

Here, we report for the first time a detailed description of the development and maturation of the digestive system of Down's herring larvae, an important component of the North Sea herring stock. The larval growth rate obtained during our experiment is similar to other experiments on Atlantic herring (Ehrlich et al. 1976; Moyano et al. 2016) and field data estimates on Downs (Denis et al. 2017). The combination of histological and enzymatic approaches allowed us to identify and discuss developmental periods at which herring larvae may be more vulnerable to fluctuating environmental conditions and suboptimal feeding conditions.

Maturation and functionality of the digestive system

Anatomical and cellular changes of the oesophagus, stomach and intestine as well as accessory glands drive the digestion processes, the efficiency of nutrients transport and absorption. Functional timing of development is the major difference between fish species and appears to be genetically programmed (Zambonino Infante and Cahu 2001).

Oesophagus

In teleosts, the alimentary canal generally develops from an undifferentiated straight tube at the yolk-sac stage to a segmented one where the oesophagus, the stomach and the intestine are differentiated. For Down's herring larvae, at 3 dph, all digestive organs, including the liver and pancreas were already distinguishable. With the opening of the mouth at around 3 dph, presence of

467 microplankton in the gut was observed before yolk sac resorption in agreement with previous
468 observations (Checkley 1982; Munk 1992). Busch (1996) showed that the first feeding, on prey larger
469 than phytoplankton cells, is allowed by the decreasing of the yolk sac diameter. He argued that the
470 oesophagus diameter increases by mechanical action in response to a decrease in the pressure
471 exerted by the yolk sac when it is consumed. Consumption of larger preys is also facilitated by the
472 presence of goblet cells that play different roles but are mostly involved in the lubrication of the
473 digestive system (Zambonino Infante et al. 2008). Goblet cells can be present before the onset of
474 exogenous feeding or appear later in the development depending of the species (Lazo et al. 2011). In
475 addition, they also differ in their histochemical features. For example, in dover sole, senegal sole and
476 yellowtail flounder, oesophagus goblet cells only secrete acid mucosubstances (Zambonino Infante et
477 al. 2008), whereas a mix of neutral and acid glycoconjugates were observed for the california halibut
478 and white seabream. The nature of this secretion varies along the ontogeny of herring larvae, there is
479 an initial production of acid mucus during stage 1 and 2 and an increased production of neutral
480 mucosubstances for stages 3 and 4. The changes in histochemical properties of the glycoconjugates is
481 species-specific, for the siberian sturgeon goblet cells in the larval oesophagus first started to
482 produce neutral glycoproteins only, and later a mix of neutral and acid mucins (Gisbert et al. 1999).
483 The mature pattern of stage 3 and stage 4 herring larvae in terms of histochemical properties is
484 similar to the one of sea bream (Sarasquete et al. 2001), with a large amount of neutral
485 mucosubstances secreted in the distal oesophageal zone and acid mucosubstances secreted in the
486 proximal oesophageal zone.

487 *Stomach*

488 The presence of pyloric ceca and gastric glands in the stomach are commonly used to histologically
489 define the transition from larvae to juvenile stage, and to an adult mode of digestion (Zambonino
490 Infante et al. 2008). No gastric glands were observed or found during the experiment, suggesting that
491 metamorphosis from a digestive point of view is not totally completed. However, the height of the
492 stomacal cells starts to increase during the stage 3 and until the end of the stage 4 (~30 mm at 69
493 dph). The stomach was the latest organ to start its development, and by 69 dph it was not fully
494 developed into the Y-shaped form typical of adult clupeids (Whitehead and Teugels 1985), allowing
495 for the ingestion of large preys.

496 *Pancreas*

497 In the exocrine pancreas, enzyme precursors are stored in the form of zymogen granules that were
498 present throughout the development of herring larvae. The most studied pancreatic enzymes are
499 alpha amylase, involved in the hydrolysis of glycogen and starch (Lazo et al. 2011), and trypsin,

considered as the most important proteolytic enzyme for fish early-life stages (Ronnestad et al. 2013). The expression pattern of these enzymes has been well described during larval development, with a lowering amylase activity, while there is an increase in trypsin activity (Zambonino Infante and Cahu 2001). In the present study, we observed the decrease in amylase activity but trypsin activity remained unchanged during the two months of herring larval development. As trypsin intestinal activity is stimulated by food ingestion (Pedersen and Hjemeland 1988), and given that we sampled in the morning before the first-feeding of the day, the stable value along the experiment might represent the basal trypsin content of larval herring. This suggests that herring larvae have an early functional exocrine pancreas, and that its maturation continues beyond 49 dph (18.5 $\pm$ 2.4 mm TL), as indicated by the decrease in amylase activity until 49 dph and the progressive change from a compact organ to a more elongate and diffuse one, like in herring adult.

Intestine

The intestine development generally corresponds to the maturation of enterocytes which is characterized by a sharp decrease in cytosolic enzymes activities in parallel to an increase in enzymes activities in brush border membranes (Zambonino Infante and Cahu 2001). These changes mark the beginning of the adult mode of digestion in fish larvae. In the present study, we assayed a set of intestinal enzymes belonging to these different enterocyte compartments. Leucine-alanine peptidase located in the cytosol, (Zambonino Infante et al. 2008) and aminopeptidase N located in the brush border (Govoni et al. 2015) ensure proteins digestion, while alkaline phosphatase is involved in the hydrolysis of phosphorylated proteins and may also be involved in amino-acids transport (Cara et al. 2003). In addition to playing a role in nutrient transport and absorption, intestinal alkaline phosphatase is a key protective enzyme in fish involved in gastrointestinal health, limiting inflammation (Lallès 2019). Ratios of brush border enzymes to cytosolic enzymes activities are largely used as indicators of intestinal maturation. In herring, the change from a larval to an adult mode of digestion in enterocytes occurred between 28 (15.0 $\pm$ 2.2 mm TL) and 35 dph (16.9 $\pm$ 2.0 mm TL), the size class and dph correspond to stage 3 larvae. The brush border structure was visible since the start of the development but was not mature until the end of the stage 3, at 35 dph when larvae were around 16 mm. In the meantime, the size of the intestine increased with more prominent villi. Stage 4 larvae were observed close to 35 dph, suggesting a synchronism between the physiological and the morphological developmental changes. As the larvae were pooled to assess enzyme activity our results reflect the general pattern of digestive maturation in herring larvae, and help to identify the moment when most of the individuals have reached this particular developmental sequence. Further analysis using other techniques (e.g. radioimmunoassay or

fluorescence technique) might be useful to assess the degree of individual variability on the maturation of the digestive system linked to ontogeny.

Critical periods and implications for Downs herring recruitment

The critical period was defined by (Hjort 1914) as the major mortality event occurring during larval development if suitable environmental conditions are not met and was hypothesized to happen at transition from endogenous to exogenous feeding (Houde 2008). In the present study, we observed that Downs herring larvae started to feed early and before the exhaustion of vitelline reserves. Moreover, liver of stages 1 and 2 larvae depicted large and numerous glycogen vacuoles showing that young herring larvae tend to develop energy storage when food is available. Glycogen plays an important role as cellular source of energy to be used later on in the development (Furukawa et al. 2018) and to cope with environmental stress like changes in food availability (Gisbert and Sarasquete 2008). Later on in the ontogeny, significant changes in liver reserves were observed. Heterogeneity in liver vacuoles size along with a decrease in glycogen was observed during stage 3 and 4, and some larvae were found with small or no vacuole. Glycogen was still present quite homogeneously but in lower quantity compared to smaller larvae and the general appearance of hepatocytes was less inflated.

Vacuoles depletion in the liver can be explained by the transition from stage 2 to stage 3 which entails a high energetic cost. Transition to stage 3 implies important morphological changes such as the development and growth of caudal and dorsal fins, as well as an increase in body height. Anatomically the organs of the digestive system also increased in size, all these changes likely being high energy demanding. These results are consistent with the decrease in growth rate observed for wild collected Downs herring larvae between 13 and 14 mm (Denis et al. 2017). Growth rate continues to decrease with the increase of larval length which may be indicative of a shift after 14 mm to a storage-oriented development strategy. Our results do not directly support this hypothesis since vacuoles in the liver were not more numerous in stage 4 larvae. These larvae are at the onset of metamorphosis, a challenging period where major physiological changes (e.g. increase in muscle development (Batty 1984), muscle fiber recruitment and surface increased sharply after 25 mm in Clyde herring (Johnston et al. 1998) also occur and require a huge amount of energy. While this could explain the depletion of liver vacuoles, change in the type of storage linked to a shift from a glycogen-based metabolism to a lipid-based one can be another hypothesis. Further analyses of larval lipid content and lipid class composition would be useful and complementary to disentangle and confirm energetic trade-offs between glycogen and lipids dynamics linked to larval ontogeny and nutritional condition.

For North Sea herring, the critical period in the wild was assessed to occur before 19 mm (Nash and Dickey-Collas 2005) since abundance of larger larvae are used to estimate the recruitment index at one year and are well correlated with abundance of age 1 herring the year after (ICES 2020). Then, rather that the transition from stage 1 to stage 2, the transition from stage 2 to 3 appears more as the critical period for North Sea herring. According to our results on the variation pattern of liver vacuoles and reserves during larval ontogeny, variability in the mortality rate experienced during this critical period may rely on the ability of larvae to make reserves before the transition, i.e. during stage 2. Stage 2 larvae in a favorable feeding environment could be able to store reserves while maintaining efficient growth, which would lead to a better potential of survival when passing to the next development stage. The use of liver reserve in stage 2 larvae as an indicator of potential of survival/mortality rate should then be investigated. Downs herring larvae, caught each year during the IBTS could be analysed using histology to estimate year to year differences in proportion of individuals with liver reserves. These results should be then correlated to annual variations in recruitment and environmental parameters. The use of the RNA/DNA ratio may easiest for long term monitoring of larval herring condition, but since it does not directly measure reserve storage, and can differ from the histological index (Di Pane et al. 2019), it first require calibration.

Conclusion

The combination of histological and histochemical techniques gave us significant insights on the ontogeny of the digestive system for Downs herring larvae. All the digestive organs were present at hatching, except for the stomach. Digestive accessory glands were even well developed before mouth opening and containing an important amount of reserves (zymogen, glycogen). The use of biochemical measurements combined with histology was complementary and indicated not only the ontogeny of cell structures but also their functionality (i.e. brush borders of the enterocytes). The fluctuations in liver reserves quantities suggest that the transition from stage 2 to 3 and the end of the stage 4, at the onset of metamorphosis into juveniles, are highly demanding in energy. These results could serve as a reference for nutritional histological condition of herring larvae from field studies. These transitions can be perceived as critical or challenging periods for larvae that have to cope with a changing environment in the wild. Our results suggest that only larvae that were able to store sufficient energy during the first-feeding stage could pass these critical periods, develop and survive. Our findings are relevant to help explain inter-annual fluctuations in Downs herring larvae survival and recruitment. Therefore, inter-annual variations in the level of energy storage (e.g. based

598 on the evaluation of the amount of vacuoles in the liver) in first-feeding stages should be examined
599 and considered as potential predictors of recruitment variability.

600

601 **Compliance with Ethical Standards**

602 **Conflict of interest** The authors declare they have no conflict of interest.

603 **Ethic approval** Fish experiments were conducted at the Ifremer-Centre de Bretagne facilities
604 (agreement number: B29-212-05) following French national regulations and authorized by the
605 Regional Ethics Committee (authorization number 16513-2018082709221792).

606 **Data availability** All data generated or analyzed during this study are included in this published
607 article.

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