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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.

81 **1. Introduction**

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

90 The economic implications of fish stock inter-annual variability have motivated research on
91 population dynamics and fish biology for more than a century (Hart and Reynolds 2002). Hjort (1914)
92 was the first to point out larval survival, and not only spawning stock biomass, as a key factor
93 determining annual recruitment and size of fish populations. He defined the transition from
94 endogenous to exogenous nutrition as the critical period ("Critical period hypothesis"), i.e. the period
95 when the larvae start feeding, and are most sensitive to starvation. This first-feeding stage has been
96 shown to be a bottleneck period for Western Baltic Sea herring, where young larvae reaching this
97 critical period before the plankton bloom contribute little to the surviving year-class (Polte et al.
98 2014). Different recruitment hypotheses based on hydrodynamic and trophodynamic factors have
99 been put forward since, and it is now well accepted that biological and physical processes act in
100 concert to determine the survival of fish at early life stages (Houde 2008), from fertilization to
101 metamorphosis and beyond. For instance, feeding success and subsequent growth rate are
102 advocated as two of the most important factors influencing larval survival and recruitment (Anderson
103 1988; Bergenius et al. 2002; Jenkins and King 2006; Robert et al. 2007).

104 Thus far, condition and physiological state of field-caught larvae have been used as indicators of
105 suitable environmental condition and potential recruitment success. These are typically measured by
106 indices, including morphometric, histological and biochemical measurements (Ferron and Leggett
107 1994). These indices are used to assess directly or indirectly the nutritional status of organisms, or to
108 infer their ability to bear starvation (Ehrlich et al. 1976). Some of them are widely used in larval fish
109 surveys, such as the relative content of RNA to DNA in tissue, or the size corrected weight of
110 individuals. Recently, Denis et al. (2017) observed that Downs herring larvae, one of the most
111 important spawning components of North Sea herring, undergo a change in their condition at around
112 13 mm. The authors observed a reduction in instantaneous growth rate (by RNA: DNA) along with an
113 increase in ingestion rate and suggested this to be a possible critical period resulting from a change in

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

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

147

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

158

159 **2. Materials and methods**

160 **2.1 Artificial fertilization**

161 Mature herring from the Downs component were obtained in November 2018 from a local
162 organization of fishermen (Coopérative Maritime Etaploise, CME) in Boulogne-Sur-Mer (France).
163 Artificial fertilization was carried out using ready-to-spawn fish at the Ifremer Manche-Mer du Nord
164 laboratory. Eggs of 27 females (mean standard length: $27.2 \pm (SD) 1.92$ cm; mean weight: $166.4 \pm$
165 32.54 g) were stripped-spawned in seawater and placed on four plastic plates (PVC) of 490 cm^2 each
166 before being fertilized with the sperm of 9 males (mean standard length: 28.0 ± 3.84 cm; mean
167 weight: 179.2 ± 32.0 g). The eggs and sperm were incubated for 10 minutes at 13.5°C and 32.7 psu,
168 before rinsing. To ensure genetic diversity, on each plate eggs from at least six females were spread
169 and the mixed sperm of at least two males was added for the fertilization. Along with the four PVC
170 plates a smaller slide of 21 cm^2 was used to monitor closely the fertilization and eggs development
171 without having to take out of water the eggs intended for the experiment. Eggs were stripped-
172 spawned on the slide and fertilized with the same protocol in order to give us an approximation on
173 fertilization success. The fertilization success of the slide was of 42%. Around 200 eggs were laid on
174 21 cm^2 , thus we can estimate that approximately 4600 eggs were present on each plate of the
175 experiment.

176 The plates were transported to Ifremer-Centre de Bretagne laboratory in boxes with oxygenated and
177 thermoregulated sea water (variation range: 13.5 to 15°C). Eggs were then incubated in a 200 L tank
178 in the dark. For the entire experiment, natural seawater was pumped from the Bay of Brest (32.5
179 psu, pH 8.0), passed through a sand filter, heated to avoid natural temperature fluctuations, filtered
180 ($2 \mu\text{m}$) and UV sterilized (PZ50, 75W, Ocene, France).

181 2.2 Larval rearing

182 Most of the eggs hatched on the 9th day of incubation (starting point of the experiment: 1 dph) and
183 larvae were kept until 69 days post hatch (dph). Three days after hatching (3 dph) about 4500 larvae
184 were counted and distributed equally in three 38 litres conical black tanks (1500 larvae by tank),
185 constituting the replicates of the experiment. A continuous flow-through system of 20 L h⁻¹ was used
186 and the temperature was set to 13°C (13.3 ± 0.25°C). Oxygen saturation was measured daily with an
187 oximeter (WTW Oxi 340, Bioblock scientific) and was always higher than 98%. In order to prevent any
188 food limitation, the daily food quantity was distributed four times during the day to maintain an *ad*
189 *libitum* level, ensuring that there were always prey in the tank during the day. To be sure to allow
190 proper feeding through time we used an increasing range of living prey sizes from phytoplankton to
191 24 h old nauplii, before weaning with feed granules.

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

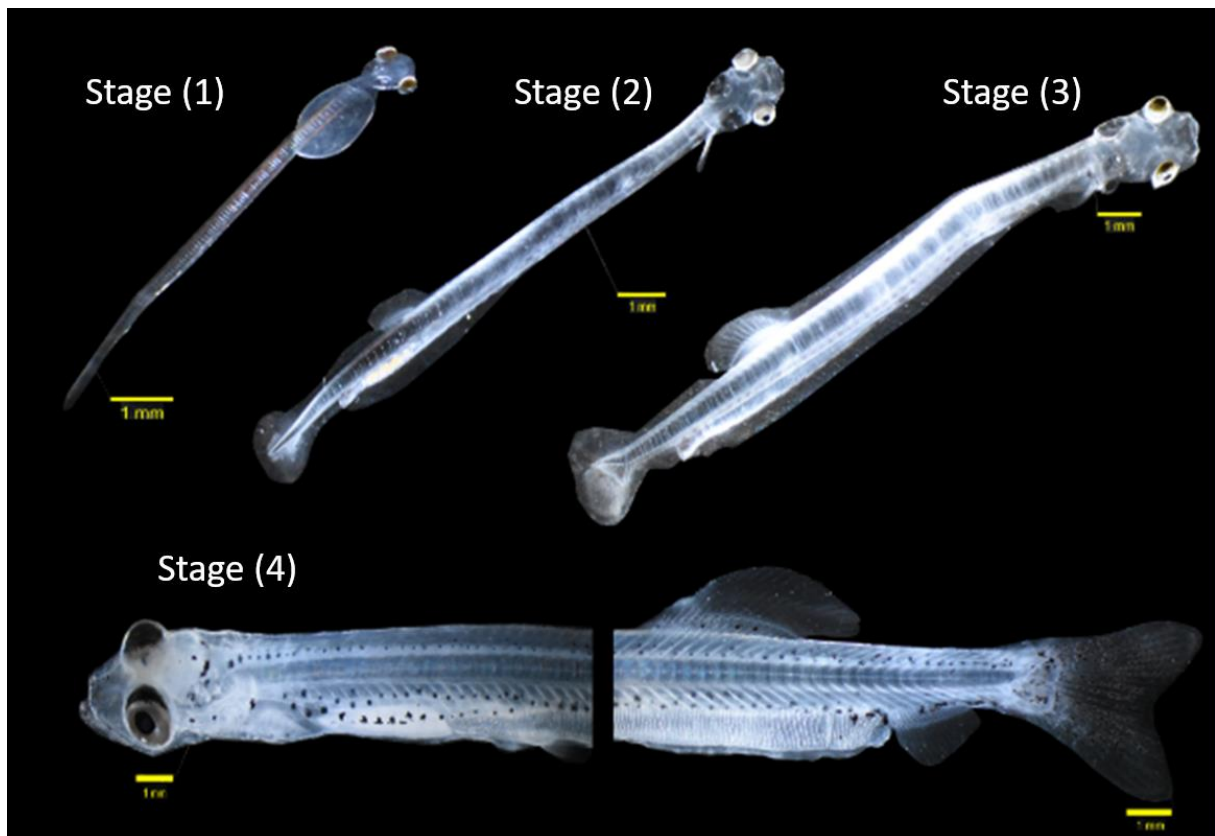
200 The feeding protocol of Moyano et al. (2016) was followed for early feeding. Yolk sac larvae were fed
201 the microalgae *R. salina* (mean: 30 000 cells mL⁻¹) and the heterotrophic protist *Oxyrrhis marina*
202 (mean: 250 cells mL⁻¹). Two days after mouth opening, *Artemia salina* nauplii (A0) were supplied in
203 the tanks (mean: 70 A0 larvae⁻¹ d⁻¹). From 22 to 39 dph a mix of *A. salina* nauplii (mean: 140 A0
204 larvae⁻¹ d⁻¹) and 24 h old enriched artemii (mean: 140 A1 larvae⁻¹ d⁻¹) were added. After that, A1 were
205 supplied in decreasing quantities (mean: 200 A1 larvae⁻¹ d⁻¹) while introducing feed granules (Neo
206 Supra, Le Gouessant, 58% proteins, 13% lipids) until 49 dph (from 0.2 to 0.5 g tank⁻¹d⁻¹). From 49 to
207 69 dph, larvae were fed only with the granules (1 g tank⁻¹ d⁻¹). The lighting sequence of the tanks was
208 modeled on aquaculture protocols. The lighting period was of 24 h a day, progressively increased
209 from 1 to 59 lux along the development, in order to maximize larval feeding, in particular for young
210 herring larvae which possess only cone cells prior to metamorphosis (Bell and Dick 1993).

211 2.3 Larval sampling

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

214 enzyme analysis. Larvae were collected with a sieve in the morning, before feeding, and euthanized
215 in ice water. As larvae are fragile organisms, homogeneous sampling through the tanks inevitably
216 leads to a slight increase in mortality. Thus, for histological individual analysis we decided to limit the
217 number of larvae sampled at the beginning of development and to increase it progressively according
218 to the progress and success of the rearing. The samplings were adjusted to not go below a minimum,
219 non-stressful, density of 25-30 larvae.L⁻¹ per tank. Four vitelline larvae were directly sampled from
220 the hatching tank at 3 dph. After that, from 2 to 13 larvae per tank were sampled at 12, 19, 21, 25,
221 28, 35, 49 and 69 dph. The larvae were preserved for 48 h in Bouin's solution, then rinsed and
222 preserved in 70% ethanol in the dark until analysis. To describe the morphological development,
223 larval stage and size were defined under binocular observations for 4 larvae (at 3 dph) + ((2-10
224 larvae) * 7 (sampling dates) * 3 (replicates)) + (13 larvae * 3 (replicates)) = 169 larvae. To have a
225 reference value on the level of shrinkage of the conservation technique, larvae sampled at 69 dph
226 were measure before and after conditioning (13 larvae per tank). A total of 35 larvae were used to
227 histologically describe the digestive system development through time.

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



231

232 **Fig. 1** Developmental stages of herring larvae: (1) yolk sac stage – 3 dph, 8-9 mm TL , (2) pre-flexion
 233 stage – 12 dph, 9-14 mm TL, (3) flexion stage – 28 dph, 12-18 mm TL and (4) post-flexion stage, the
 234 larvae is cut in two parts because it was unfortunately too long to fit entirely under the binocular –
 235 69 dph, 15-30 mm TL, binocular observations

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

240 **2.4 Organogenesis of the digestive system**

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

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

Organs	Tissue	Features		
<i>Oesophagus</i>	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
<i>Rudimentary Stomach</i>	Epithelium	simple	pseudo-stratified	stratified
	Epithelial cells shape	cubic		prismatic
	Villi	present		absent
<i>Differentiated Stomach</i>	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
<i>Rudimentary Intestine</i>	Epithelial cells shape	prismatic		
	Goblet cells	present		absent
	Mucins	acid	neutral	both
	Villi	present		absent
	Brush Border	present		absent
<i>Differentiated intestine (anterior and posterior)</i>	Epithelial cells shape	prismatic		
	Goblet cells	present		absent
	Mucins	acid	neutral	both
	Villi	moderate		prominent
	Brush Border	present		absent
	Vacuoles	present		absent
<i>Exocrine Pancreas</i>	Acini stain	dark		pale
	Tissue organisation	Indistinct acini, detachments		Distinct acini, contiguous
	Zymogen	present		absent
<i>Endocrine Pancreas</i>	Langerhans islets	present, number		absent
<i>Liver</i>	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

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

297 3. Results

298 3.1 Size distribution during developmental stages

299 LMEM fixed effect estimate of the variation of length over time was of 0.22 mm.d⁻¹ (Table 2).

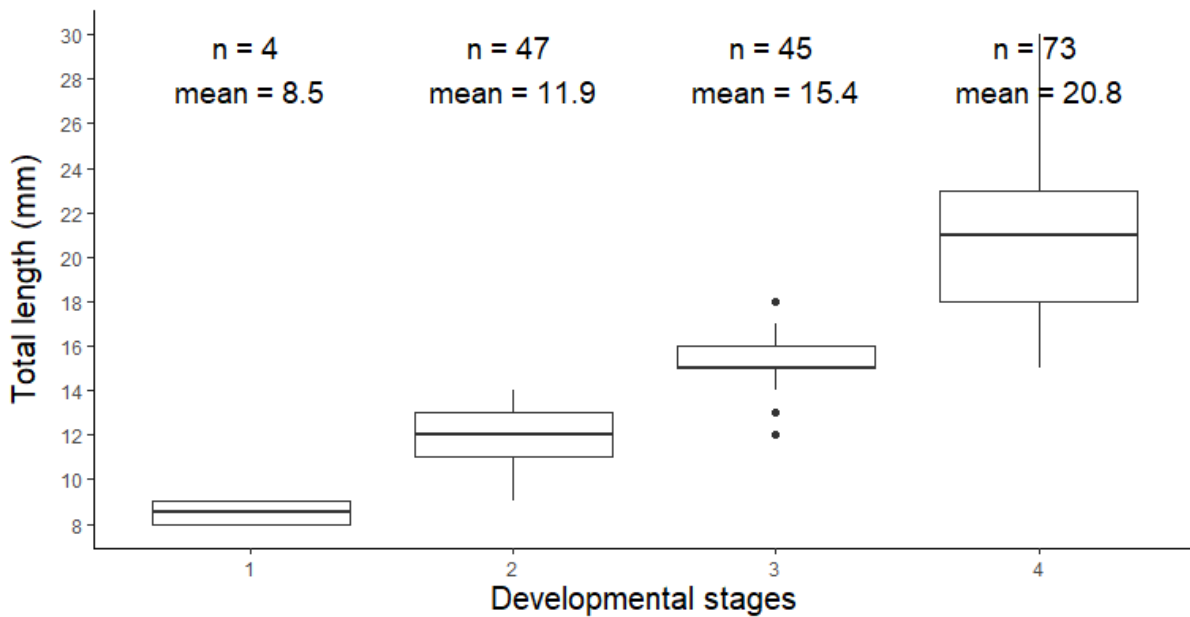
300 **Table 2** Estimated model fixed effects of the variation of length over time using "tank" as a random
301 effect for 761 observations.

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

302

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

307



308

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

310 Additional to size measurements, the developmental stage was determined for 169 larvae sampled
 311 for histological analysis. Size ranges between each stage are well separated (Fig. 2). Stage 1 larvae
 312 were found at 3 dph, the larvae were thin and long with a straight notochord, had a visible intestine
 313 and vitelline reserves. In stage 2 larvae between 12 and 35 dph, the vitellus was totally resorbed and
 314 the caudal and dorsal fins started to differentiate. For stage 3 larvae between 21 and 35 dph, body
 315 height was higher, the intestine bigger and the caudal, dorsal and anal fins fully differentiated.
 316 Melanophores were also present along the superior part of the intestine and some above the head
 317 and on the caudal fin. For stage 4 larvae from 35 to 69 dph, all the body features were bigger, the
 318 whole body was thicker and the pelvic fins started to differentiate. Melanophores were present all
 319 over the body and concentrated above the intestine, on the dorsal part of the body, above the head,
 320 around the mouth and on the dorsal fin. The measurements came from individuals stored
 321 successively in Bouin's solution and 70% ethanol for histology. The conditioning method can result in
 322 shrinkage of the larvae. Individuals measured before and after conservation shrank by an average of
 323 11.2 % (± 7.4 %, n = 39).

324 **3.2 Digestive system development**

325 **Yolk-sac stage (stage 1)**

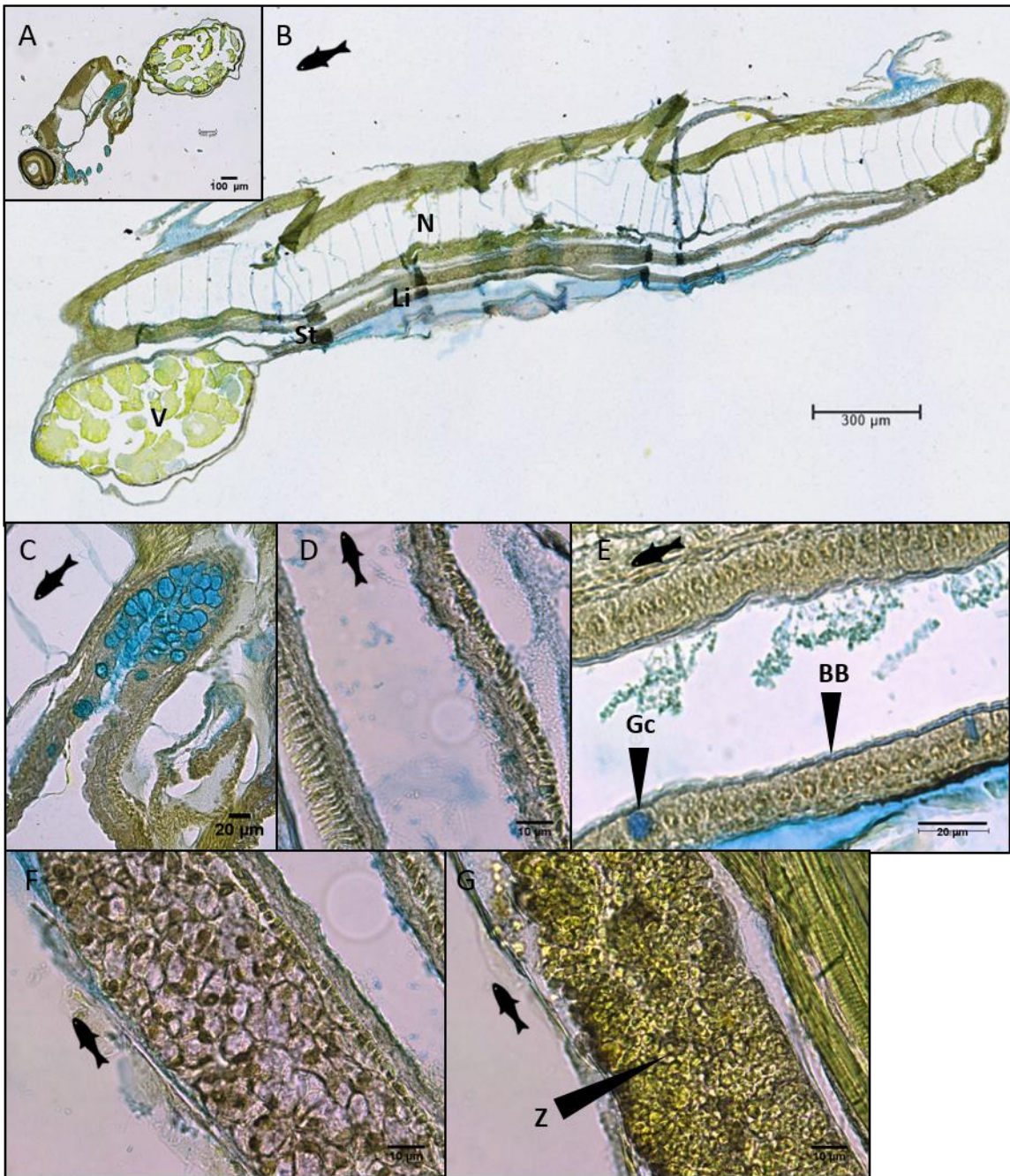
326 At 3 dph, larvae were around 8 mm and the mouth was open with the yolk sac still present. The
 327 oesophagus was relatively short, narrow and folded, with an important amount of goblet cells (Fig.
 328 3c). Only acid mucosubstances (AB positive, Fig. 4a) were detected. The stomach was not yet

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

337

338

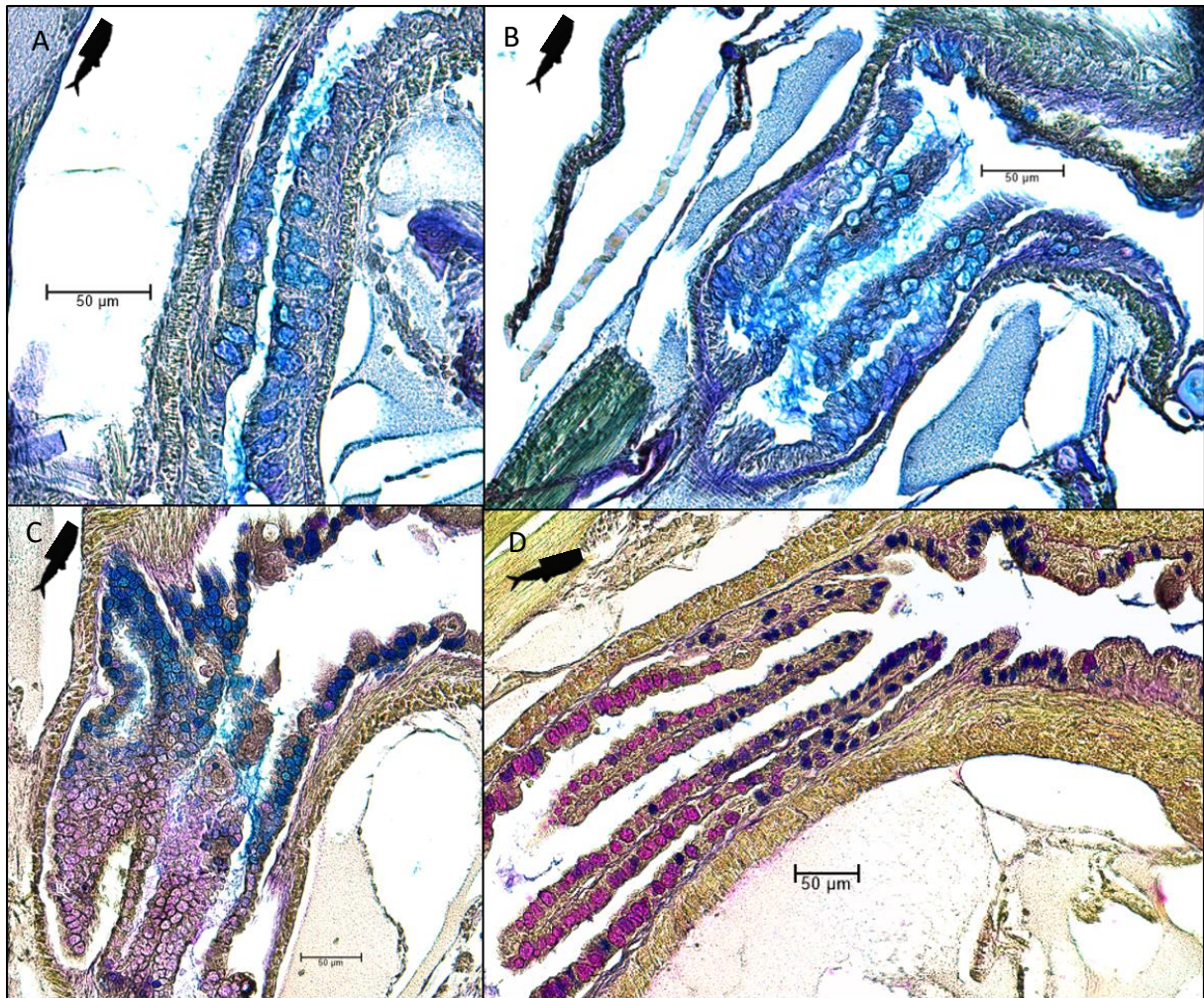
339



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

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



351
352 **Fig. 4** – Oesophagus sagittal sections of Downs herring larvae at stage 1 (A), stage 2 (B), stage 3 (C)
353 and stage 4 (D), colored with periodic acid-Schiff, alcian blue pH2.5, Groat's hematoxylin and picro-
354 indigo carmine. Magnification x200, scale bar = 50 µm, the black fish icon represents the orientation
355 of the slide to help locate the head and the tail position

356

357 **Pre-flexion stage (Stage 2)**

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

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

369 **Flexion stage (Stage 3)**

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

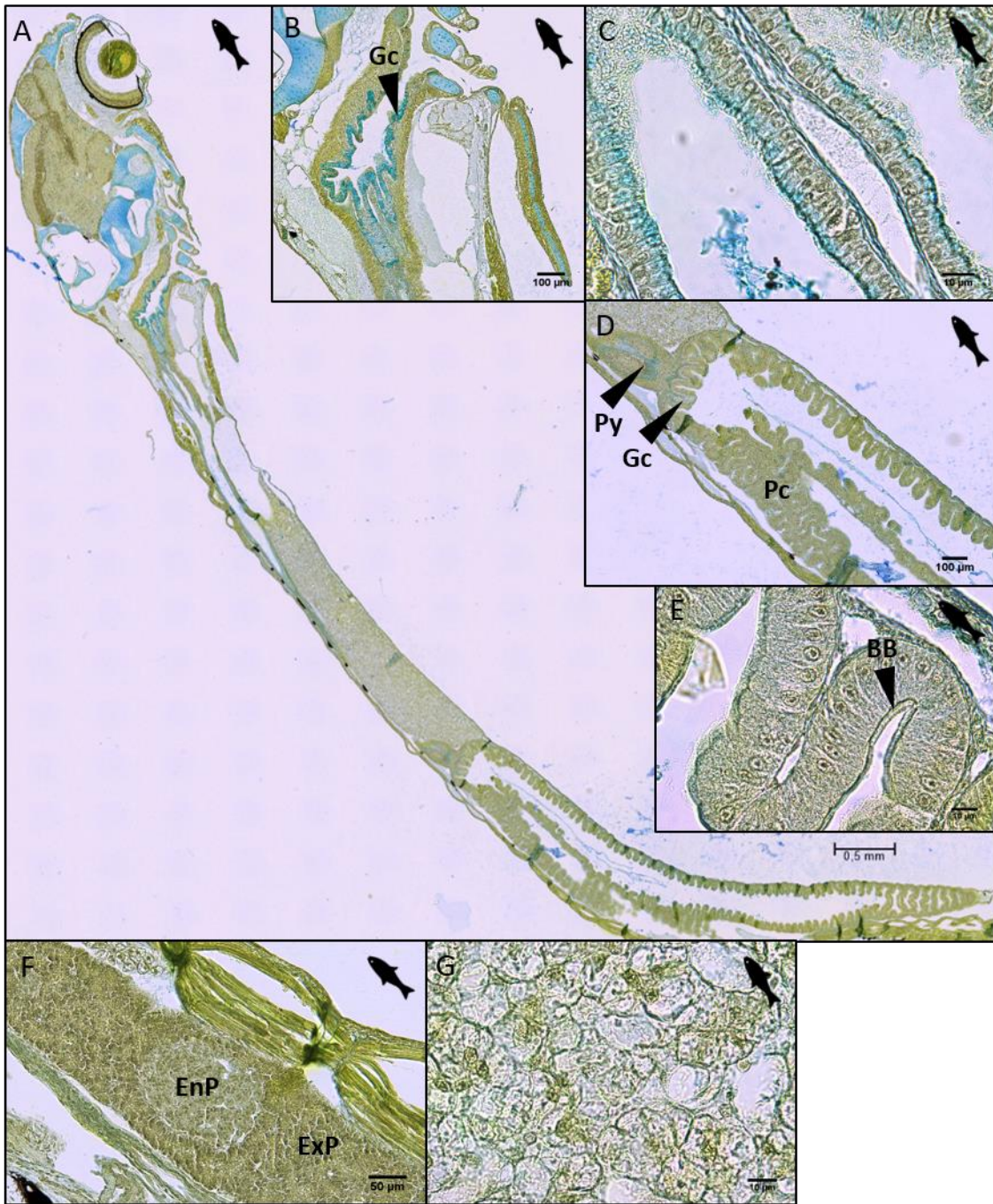
384 **Post-flexion stage (Stage 4)**

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

396 5e,d). At 49 dph the acini were still distinct but at 69 dph the structure of the exocrine pancreas was
397 more diffuse. At the end of the stage 4, one large endocrine islet was still present in the middle of
398 the pancreas and small islets were observed on either side of the larger one (Fig. 5f). The pancreas
399 expanded further along the intestine. The liver of stage 4 larvae, like for stage 3, was highly variable
400 with some individuals presenting numerous and large vacuoles (Fig. 5g, 6a) while for others the
401 vacuoles and amount of glycogen were reduced or absent (Fig. 6b,c).

402

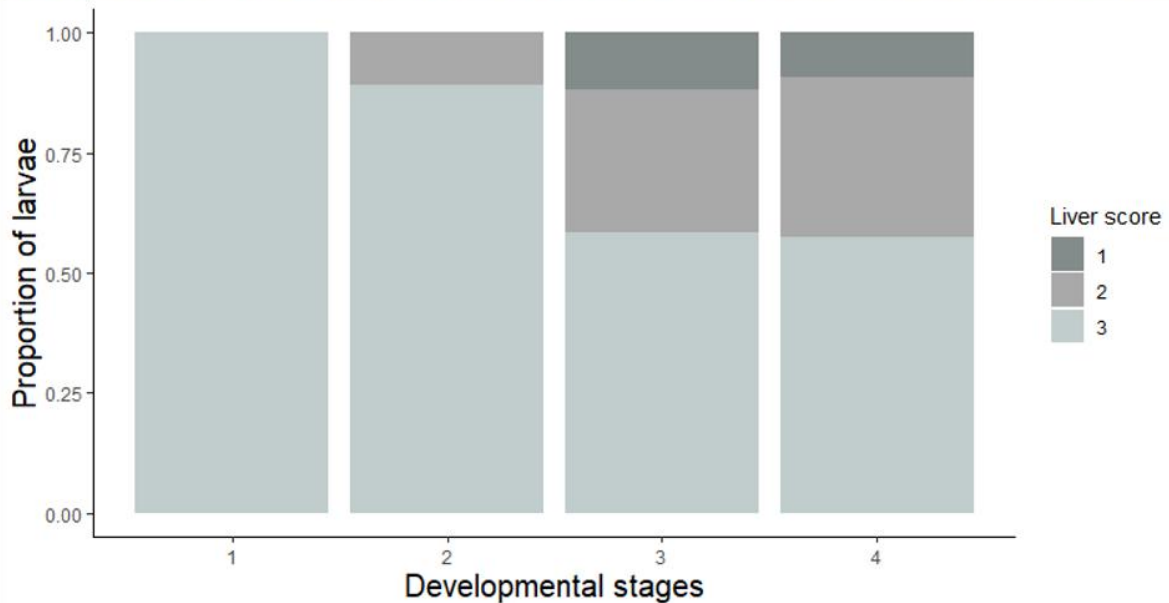
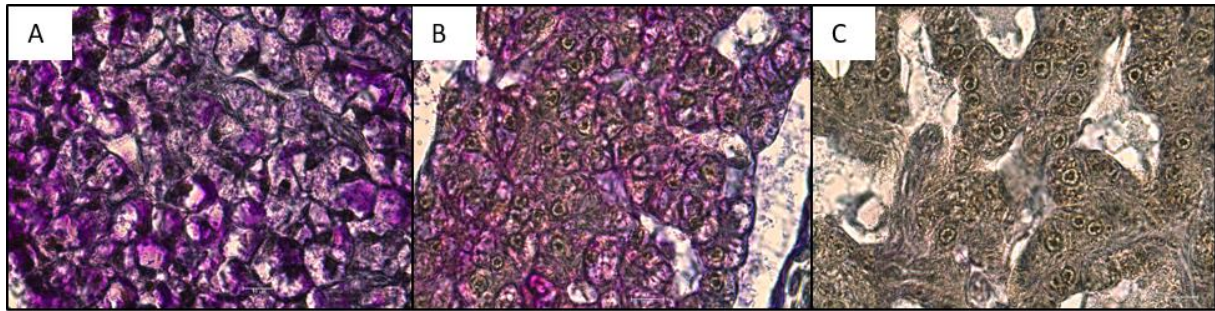
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404

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 caeca, 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

413 = 50 μm . **G** Details of the liver with large cells and vacuoles, and a small amount of glycogen (pink),
 414 Magnification x1000; oil immersion, scale bar = 10 μm . Abbreviations: BB Brush Borders, Gc Goblet
 415 cells, I Intestine, Li Liver, N Notochord, Pancreas with ExP Exocrine Pancreas and EnP Endocrine
 416 Pancreas, Oe Oesophagus, Pc Pyloric ceca, Py Pylorus, Sb Swim bladder, St stomach, V Vitellus Z
 417 Zymogen granules within acinar cells, the black fish icon represents the orientation of the slide to
 418 help locate the head and the tail position



419

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

424 3.3 Activity of digestive enzymes

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

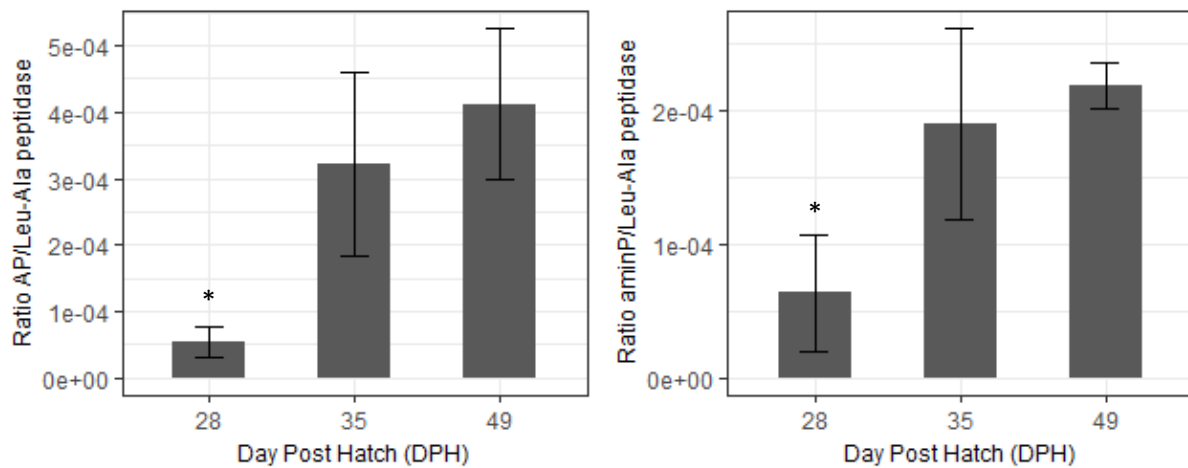
428 3). Amylase specific activity was different through time (ANOVA, $F(4,10) = 12.14$, $P < 0.01$), a post
 429 hoc Tukey test showed that the activity was higher at 14 dph ($P < 0.05$) and decreased later from
 430 5079 ± 470.6 to a mean of 818.1 ± 324.7 mU mg protein⁻¹ at 49 dph. Intestinal enzymes specific
 431 activities were tested for larvae at 28, 35 and 49 dph. No statistical significant differences were found
 432 for the intestinal enzymes specific activities Aminopeptidase N (ANOVA, $F(2, 6) = 2.79$, $P = 0.139$),
 433 Alkaline phosphatase (ANOVA, $F(2, 6) = 4.80$, $P = 0.057$) and Leucine-alanine peptidase (ANOVA, $F(2,$
 434 $6) = 0.637$, $P = 0.561$), at the three different ages. The evolution of the ratio between segmental
 435 activities of the brush border and cytosolic enzymes differed through time (AP/Leu-Ala: ANOVA, F
 436 $(2,6) = 9.55$, $P = 0.014$; AminP/Leu-Ala: ANOVA, $F(2,6) = 8.37$, $P = 0.018$), a post hoc Tukey test
 437 highlighted a shift between 28 and 35-49 dph (Fig. 7, $P < 0.05$).

438

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

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

443



444

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

449 **4. Discussion**

450 Here, we report for the first time a detailed description of the development and maturation of the
 451 digestive system of Down's herring larvae, an important component of the North Sea herring stock.
 452 The larval growth rate obtained during our experiment is similar to other experiments on Atlantic
 453 herring (Ehrlich et al. 1976; Moyano et al. 2016) and field data estimates on Downs (Denis et al.
 454 2017). The combination of histological and enzymatic approaches allowed us to identify and discuss
 455 developmental periods at which herring larvae may be more vulnerable to fluctuating environmental
 456 conditions and suboptimal feeding conditions.

457 **Maturation and functionality of the digestive system**

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

462 *Oesophagus*

463 In teleosts, the alimentary canal generally develops from an undifferentiated straight tube at the
 464 yolk-sac stage to a segmented one where the oesophagus, the stomach and the intestine are
 465 differentiated. For Down's herring larvae, at 3 dph, all digestive organs, including the liver and
 466 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,

500 considered as the most important proteolytic enzyme for fish early-life stages (Ronnestad et al.
501 2013). The expression pattern of these enzymes has been well described during larval development,
502 with a lowering amylase activity, while there is an increase in trypsin activity (Zambonino Infante and
503 Cahu 2001). In the present study, we observed the decrease in amylase activity but trypsin activity
504 remained unchanged during the two months of herring larval development. As trypsin intestinal
505 activity is stimulated by food ingestion (Pedersen and Hjemeland 1988), and given that we sampled
506 in the morning before the first-feeding of the day, the stable value along the experiment might
507 represent the basal trypsin content of larval herring. This suggests that herring larvae have an early
508 functional exocrine pancreas, and that its maturation continues beyond 49 dph (18.5 ± 2.4 mm TL),
509 as indicated by the decrease in amylase activity until 49 dph and the progressive change from a
510 compact organ to a more elongate and diffuse one, like in herring adult.

511 *Intestine*

512 The intestine development generally corresponds to the maturation of enterocytes which is
513 characterized by a sharp decrease in cytosolic enzymes activities in parallel to an increase in enzymes
514 activities in brush border membranes (Zambonino Infante and Cahu 2001). These changes mark the
515 beginning of the adult mode of digestion in fish larvae. In the present study, we assayed a set of
516 intestinal enzymes belonging to these different enterocyte compartments. Leucine-alanine peptidase
517 located in the cytosol, (Zambonino Infante et al. 2008) and aminopeptidase N located in the brush
518 border (Govoni et al. 2015) ensure proteins digestion, while alkaline phosphatase is involved in the
519 hydrolysis of phosphorylated proteins and may also be involved in amino-acids transport (Cara et al.
520 2003). In addition to playing a role in nutrient transport and absorption, intestinal alkaline
521 phosphatase is a key protective enzyme in fish involved in gastrointestinal health, limiting
522 inflammation (Lallès 2019). Ratios of brush border enzymes to cytosolic enzymes activities are
523 largely used as indicators of intestinal maturation. In herring, the change from a larval to an adult
524 mode of digestion in enterocytes occurred between 28 (15.0 ± 2.2 mm TL) and 35 dph (16.9 ± 2.0
525 mm TL), the size class and dph correspond to stage 3 larvae. The brush border structure was visible
526 since the start of the development but was not mature until the end of the stage 3, at 35 dph when
527 larvae were around 16 mm. In the meantime, the size of the intestine increased with more
528 prominent villi. Stage 4 larvae were observed close to 35 dph, suggesting a synchronism between the
529 physiological and the morphological developmental changes. As the larvae were pooled to assess
530 enzyme activity our results reflect the general pattern of digestive maturation in herring larvae, and
531 help to identify the moment when most of the individuals have reached this particular
532 developmental sequence. Further analysis using other techniques (e.g. radioimmunoassay or

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

535 **Critical periods and implications for Downs herring recruitment**

536 The critical period was defined by (Hjort 1914) as the major mortality event occurring during larval
537 development if suitable environmental conditions are not met and was hypothesized to happen at
538 transition from endogenous to exogenous feeding (Houde 2008). In the present study, we observed
539 that Downs herring larvae started to feed early and before the exhaustion of vitelline reserves.
540 Moreover, liver of stages 1 and 2 larvae depicted large and numerous glycogen vacuoles showing
541 that young herring larvae tend to develop energy storage when food is available. Glycogen plays an
542 important role as cellular source of energy to be used later on in the development (Furukawa et al.
543 2018) and to cope with environmental stress like changes in food availability (Gisbert and Sarasquete
544 2008). Later on in the ontogeny, significant changes in liver reserves were observed. Heterogeneity in
545 liver vacuoles size along with a decrease in glycogen was observed during stage 3 and 4, and some
546 larvae were found with small or no vacuole. Glycogen was still present quite homogeneously but in
547 lower quantity compared to smaller larvae and the general appearance of hepatocytes was less
548 inflated.

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

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

582

583 **Conclusion**

584 The combination of histological and histochemical techniques gave us significant insights on the
585 ontogeny of the digestive system for Downs herring larvae. All the digestive organs were present at
586 hatching, except for the stomach. Digestive accessory glands were even well developed before
587 mouth opening and containing an important amount of reserves (zymogen, glycogen). The use of
588 biochemical measurements combined with histology was complementary and indicated not only the
589 ontogeny of cell structures but also their functionality (i.e. brush borders of the enterocytes). The
590 fluctuations in liver reserves quantities suggest that the transition from stage 2 to 3 and the end of
591 the stage 4, at the onset of metamorphosis into juveniles, are highly demanding in energy. These
592 results could serve as a reference for nutritional histological condition of herring larvae from field
593 studies. These transitions can be perceived as critical or challenging periods for larvae that have to
594 cope with a changing environment in the wild. Our results suggest that only larvae that were able to
595 store sufficient energy during the first-feeding stage could pass these critical periods, develop and
596 survive. Our findings are relevant to help explain inter-annual fluctuations in Downs herring larvae
597 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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620 **References**

621 Anderson JT (1988) A Review of Size Dependant Survival During Pre-recruit Stages of Fishes in
622 Relation to Recruitment. J Northwest Atl Fish Sci 8:55–66. doi: 10.2960/J.v8.a6

623 Barr DJ, Levy R, Scheepers C, Tily HJ (2013) Random effects structure for confirmatory hypothesis
624 testing: Keep it maximal. J Mem Lang 68:255–278. doi: 10.1016/j.jml.2012.11.001

625 Bates D, Mächler M, Bolker BM, Walker SC (2015) Fitting linear mixed-effects models using lme4. J
626 Stat Softw. doi: 10.18637/jss.v067.i01

627 Batty RS (1984) Development of swimming movements and musculature of larval herring (*Clupea*

628 *harengus*). J Exp Biol 110:217–29.

629 Bell M V, Dick JR (1993) The appearance of rods in the eyes of herring and increased di-
630 docosahexaenoyl molecular species of phospholipids. J mar biol Ass 73:679–688.

631 Bessey OA, Lowry OH, Brock MJ (1946) Rapid coloric method for determination of alkaline
632 phosphatase in five cubic millimeters of serum.

633 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of
634 protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

635 Busch A (1996) Transition from endogenous to exogenous nutrition: Larval size parameters
636 determining the start of external feeding and size of prey ingested by Ruegen spring herring
637 *Clupea harengus*. Mar Ecol Prog Ser 130:39–46. doi: 10.3354/meps130039

638 Cahu CL, Infante JLZ (1995) Effect of the molecular form of dietary nitrogen supply in sea bass larvae:
639 Response of pancreatic enzymes and intestinal peptidases. Fish Physiol Biochem 14:209–214.
640 doi: 10.1007/BF00004311

641 Cahu CL, Zambonino Infante JL (1994) Early weaning of sea bass (*Dicentrartrus labrax*) larvae with a
642 compound diet: effect on digestive enzymes. Comp Biochem Physiol 109A:213–222.

643 Cara JB, Moyano FJ, Cárdenas S, Fernández-Díaz C, Yúfera M (2003) Assessment of digestive enzyme
644 activities during larval development of white bream. J Fish Biol 63:48–58. doi: 10.1046/j.1095-
645 8649.2003.00120.x

646 Checkley D (1982) Selective feeding by Atlantic herring (*Clupea harengus*) larvae on zooplankton in
647 natural assemblages. Mar Ecol Prog Ser 9:245–253.

648 Crane RK, Boge G, Rigal A (1979) Isolation of brush border membranes in vesicular form from the
649 intestinal spiral valve of the small digfish (*Scyliorhinus canicula*). Biochim Biophys Acta
650 554:264–267.

651 Denis J, Mahe K, Tavernier E, Monchy S, Vincent D, Vallet C, Marchal P, Antajan E, Caboche J,
652 Lefebvre V, Cordier R, Loots C (2017) Ontogenetic changes in the larval condition of Downs
653 herring: use of a multi-index approach at an individual scale. Mar Biol 164:1–14. doi:
654 10.1007/s00227-017-3180-3

655 Di Pane J, Joly L, Koubbi P, Giraldo C, Monchy S, Tavernier E, Marchal P, Loots C (2019) Ontogenetic
656 shift in the energy allocation strategy and physiological condition of larval plaice (*Pleuronectes*
657 *platessa*). PLoS One 14:e0222261. doi: 10.1371/journal.pone.0222261

658 Di Pane J, Gendrot F, Giraldo C, Marchal P, Koubbi P, Loots C (2020) Evaluating the histological-based
659 condition of wild collected larval fish: A synthetic approach applied to common sole (*Solea*
660 *solea*). J Mar Syst 204:103309. doi: 10.1016/j.jmarsys.2020.103309

661 Dickey-Collas M, Nash RDM, Brunel T, Van Damme CJG, Marshall CT, Payne MR, Corten A, Geffen AJ,
662 Peck MA, Hatfield EMC, Hintzen NT, Enberg K, Kell LT, Simmonds EJ (2010) Lessons learned from
663 stock collapse and recovery of North Sea herring: A review. ICES J Mar Sci 67:1875–1886. doi:
664 10.1093/icesjms/fsq033

665 Doyle MJ (1977) A Morphological Staging System for the Larval Development of the Herring . J Mar
666 Biol Assess 57:859–867.

667 Ehrlich KF, Blaxter JHS, Pemberton R (1976) Morphological and histological changes during the
668 growth and starvation of herring and plaice larvae. Mar Biol 35:105–118. doi:
669 10.1007/BF00390932

670 Ferron A, Leggett WC (1994) An appraisal of condition measures for marine fish larvae. Adv Mar Biol
671 30:217–303.

672 Foley CJ, Bradley DL, Höök TO (2016) A review and assessment of the potential use of RNA:DNA
673 ratios to assess the condition of entrained fish larvae. Ecol Indic 60:346–357. doi:
674 10.1016/j.ecolind.2015.07.005

675 Furukawa F, Irachi S, Koyama M, Baba O, Akimoto H, Okumura S ichi, Kagawa H, Uchida K (2018)
676 Changes in glycogen concentration and gene expression levels of glycogen-metabolizing
677 enzymes in muscle and liver of developing masu salmon. Comp Biochem Physiol -Part A Mol
678 Integr Physiol 225:74–82. doi: 10.1016/j.cbpa.2018.07.003

679 Gisbert E, Sarasquete C (2008) Nutritional cellular biomarkers in early life stages of fish. Histol
680 Histopathol 23:1525–1539.

681 Gisbert E, Sarasquete MC, Williot P, Castello-Orvay F (1999) Histochemistry of the development of
682 the digestive system of Siberian sturgeon during early ontogeny. J Fish Biol 55:596–616.

683 Govoni JJ, Boehlert GW, Watanabe Y (2015) The physiology of digestion in fish The physiology of
684 digestion in fish larvae. doi: 10.1007/BF00005160

685 Hart PJB, Reynolds JD (2002) Handbook of Fish Biology and Fisheries Volume 2. Blackwell Science Ltd

686 Hjort J (1914) Fluctuations in the great fisheries of northern Europe viewed in the light of biological
687 research. ICES J Mar Sci 20:1–228.

688 Holm H, Hanssen LE, Krogdahlf ÅS, Florholm A (1988) Metabolism and Hormonal Regulation High
689 and Low Inhibitor Soybean Meals Affect Human Duodenal Proteinase Activity Differently : In
690 Vivo Comparison with Bovine Serum Albumin. J Nutr 515–520.

691 Houde ED (2008) Emerging from Hjort’s shadow. J Northwest Atl Fish Sci 41:53–70. doi:
692 10.2960/J.v41.m634

693 ICES (2020) Herring Assessment Working Group for the Area South of 62° N (HAWG).

694 Johnston IA, Cole NJ, Abercromby M, Vieira VLA (1998) Embryonic temperature modulates muscle
695 growth characteristics in larval and juvenile herring. J Exp Biol 201:623–646.

696 Kuznetsova A, Brockhoff PB, Christensen RHB (2017) lmerTest Package: Tests in Linear Mixed Effects
697 Models . J Stat Softw. doi: 10.18637/jss.v082.i13

698 Lallès JP (2019) Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny,
699 and environmental and nutritional modulation. Rev Aquac 1–27. doi: 10.1111/raq.12340

700 Lazo J, Darias M, Gisbert E (2011) Ontogeny of the Digestive Tract. In: G. Joan H (ed) Larval Fish
701 Nutrition, Wiley-Blac. John Wiley & Sons, Inc, pp 5–46

702 Maroux S, Louvard D, Baratti J (1973) The aminopeptidase from hog-intestinal brush border.

703 Métais P, Bieth J (1968) Détermination de l’ α -amylase par une microtechnique. Ann Biol Clin 26:133–
704 142.

705 Moyano M, Illing B, Peschutter P, Huebert KB, Peck MA (2016) Thermal impacts on the growth,
706 development and ontogeny of critical swimming speed in Atlantic herring larvae. Comp
707 Biochem Physiol -Part A Mol Integr Physiol 197:23–34. doi: 10.1016/j.cbpa.2016.02.020

708 Munk P (1992) Foraging behaviour and prey size spectra of larval herring *Clupea harengus*. Mar Ecol
709 Prog Ser 80:149–158. doi: 10.3354/meps080149

710 Nash RDM, Dickey-Collas M (2005) The influence of life history dynamics and environment on the
711 determination of year class strength in North Sea herring (*Clupea harengus* L.). Fish Oceanogr
712 14:279–291. doi: 10.1111/j.1365-2419.2005.00336.x

713 Nicholson JA, Kim S (1975) A One-Step for Intestinal Peptide Acid Oxidase Assay Hydrolase Activity A
714 number of methods have been described for the assay of intestinal peptide hydrolase activity .
715 The spectrophotometric assay described by Josefsson and Lindberg (1) is not suitable. Anal
716 Biochem 117:110–117.

717 Payne MR, Hatfield EMC, Dickey-Collas M, Falkenhaus T, Gallego A, Gröger J, Licandro P, Llope M,
718 Munk P, Röckmann C, Schmidt JO, Nash RDM (2009) Recruitment in a changing environment:
719 The 2000s North Sea herring recruitment failure. ICES J Mar Sci 66:272–277. doi:
720 10.1093/icesjms/fsn211

721 Pedersen BH, Hjemeland K (1988) Fate of trypsin and assimilation efficiency in larval herring (*Clupea*
722 *harengus*) following digestion of copepods. Mar Biol 97:467–476.

723 Pedersen BH, Nilssen EM, Hjelmeland K (1987) Variations in the content of trypsin and trypsinogen in
724 larval herring (*Clupea harengus*) digesting copepod nauplii. Mar Biol 181:171–181.

725 Polte P, Kotterba P, Hammer C, Gröhsler T (2014) Survival bottlenecks in the early ontogenesis of
726 Atlantic herring (*Clupea harengus*, L.) in coastal lagoon spawning areas of the western Baltic
727 Sea. ICES J Mar Sci 71:982–990. doi: 10.4135/9781412953924.n678

728 Ronnestad I, Manuel Y, Ueberschar B, Ribeiro L, Sæle O, Boglione C (2013) Feeding behaviour and
729 digestive physiology in larval fish : current knowledge , and gaps and bottlenecks in research.
730 Rev Aquac 5:S59–S98. doi: 10.1111/raq.12010

731 Sarasquete C, Gisbert E, Ribeiro L, Vieira L, Dinis MT (2001) Glyconjugates in epidermal, branchial and
732 digestive mucous cells and gastric glands of gilthead sea bream, *Sparus aurata*, Senegal sole,
733 *Solea senegalensis* and Siberian sturgeon, *Acipenser baeri* development. Eur J Histochem
734 45:267–278. doi: 10.4081/1637

735 Suthers I (2000) Significance of larval condition: comment on laboratory experiments. Can J Fish
736 Aquat Sci 57:1534–1536. doi: 10.1139/f00-098

737 Ueberschär B, Clemmesen C (1992) A comparison of the nutritional condition of herring larvae as
738 determined by two biochemical methods - tryptic enzyme activity and RNA/DNA ratio
739 measurements. ICES J Mar Sci 49:245–249.

740 Whitehead PJP, Teugels GG (1985) The West African Pygmy Herring *Sierrathrissa leonensis*: General
741 features, Visceral Anatomy, and Osteology. Am museum Novit 2835:1–44. doi: 10.2307/772463

742 Zambonino Infante JL, Cahu CL (2001) Ontogeny of the gastrointestinal tract of marine fish larvae.
743 Comp Biochem Physiol 130:477–487.

744 Zambonino Infante JL, Cahu CL, Peres A (1997) Partial Substitution of Di- and Tripeptides for Native
745 Proteins in Sea Bass Diet Improves *Dicentrarchus labrax* Larval Development 1. Am Soc Nutr Sci
746 608–614.

747 Zambonino Infante JL, Gisbert E, Sarasquete C, Navarro I, Gutiérrez J, Cahu CL (2008) Ontogeny and
748 physiology of the digestive system of marine fish larvae. In: Feeding and Digestive Functions of
749 Fishes. pp 281–348
750