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1 **Do environmental conditions (temperature and food composition) affect otolith shape during fish**
2 **early-juvenile phase? An experimental approach applied to European Seabass (*Dicentrarchus***
3 ***labrax*)**

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20 **Abstract**

21 Otolith shape is an efficient tool for fish stock discrimination. Therefore, it is crucial to
22 identify the environmental parameters that could influence otolith morphogenesis and growth.
23 Current global ocean warming negatively affects the production of polyunsaturated fatty acids
24 (EPA and DHA) by phytoplankton and thus their availability in marine food webs. As EPA
25 and DHA are essential nutrients for most fish species, their deficiency could affect many
26 aspects of fish physiology, notably otolith morphogenesis. The goal of this study was to
27 assess experimentally the combined influences of temperature (T) and dietary polyunsaturated
28 fatty acid (PUFA) content on European seabass (*Dicentrarchus labrax*) otolith morphogenesis
29 during early life stages. 300 individuals were reared from 94 to 200 days post hatch (dph) at
30 15 or 20°C and fed a high (1.65% PUFA) or low (0.73% PUFA) nutrient diet. To analyze the
31 temperature effect, Growing Degree Days (GDD) were used. Otolith morphometric
32 measurements, shape factors and normalized Elliptical Fourier coefficients describing outline
33 shape were extracted using image analysis. The directional asymmetry (DA) between left and
34 right otolith shapes was not significantly correlated to the GDD or food nutrient level. The
35 effects of T and PUFA on otolith shape were estimated using four linear mixed effects models
36 and, for all descriptors of otolith outline, the best model included GDD, PUFA and T and their
37 interaction as predictors. This temperature effect was described by GDD value and the

38 interaction between GDD and T. Consequently, temperature has an effect on the speed but
39 also on the morphogenesis trajectory of the otolith. Conversely, the concentration of PUFA in
40 the diet had no significant effect on otolith shape. The otolith shape was first modified by a
41 decrease in the width/length ratio during the first period of experiment and by the distance
42 between rostrum and antirostrum during the second period of experiment. Our results indicate
43 that otolith shape is very sensitive to environmental temperature and, as such, can be an
44 effective tool for identifying fish stocks that have experienced different temperature regimes.

45 Keywords : n-3 polyunsaturated fatty acids, otolith shape, temperature effect, elliptic Fourier
46 descriptors, multivariate mixed-effects models, directional asymmetry

47 *1. Introduction*

48 Climate change involves principally an increase of sea surface temperature of 0.74°C during
49 the last century that is expected to be followed by a further increase of about 1.5 °C by the
50 end of the century (Allen et al., 2018). Several studies have shown that elevated temperatures
51 may significantly influence whole ecosystems and affect animals' physiology in relation to
52 their thermal optima or tolerance (Thomas et al., 2004; Mieszkowska et al., 2006; Hofmann
53 and Todgham, 2010; Pörtner, 2010).

54 Global climate change also induces ocean acidification and deoxygenation episodes (Diaz,
55 2001; Caldeira and Wickett, 2003; Pörtner and Knust, 2007; Feely et al., 2008; Rabalais et al.,
56 2009). These environmental changes could reduce and modify the base of marine food webs,
57 notably phytoplankton species assemblages, with potential negative consequences on the
58 production of omega-3 polyunsaturated fatty acids (n-3 PUFA) (Ackman and Tocher 1968;
59 Thompson et al., 1992; Renaud et al. 2002; Guschina and Harwood 2006; Capuzzo et al.,
60 2017). Fatty acids are the primary constituents of polar and neutral lipids, specifically
61 phospholipids and triacylglycerols, respectively. N-3 PUFA, particularly eicosapentaenoic
62 acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), contained in phospholipids
63 (Henderson and Tocher, 1987), are exclusively synthesized by phytoplankton species. There
64 is a negative correlation between temperature and the production of n-3 PUFA by
65 phytoplankton. n-3 PUFA have a critical role as the primary building blocks of cell

66 membranes as components of phospholipids, according to the concept of homeoviscous
67 adaptation (i.e. adjustment of the n-3 PUFA to ensure the viscosity and membrane exchanges
68 necessary for the proper functioning of the organism; Ernst et al., 2016). N-3 PUFA are
69 essential fatty acids (EFA) that animals, including fish, must ingest as they are almost
70 exclusively produced by phytoplankton and can rarely be synthesized *de novo* by the animals
71 themselves (Brett and Müller-Navarra, 2003). A climate-change-induced reduction in dietary
72 PUFA availability could thus impact fish physiology by changing cell membrane fluidity, the
73 production of eicosanoids and main physiological processes (growth, respiration and
74 reproductive rates) with potential consequences on fish recruitment (Brett and Müller-
75 Navarra, 2003). For example, in European seabass (*Dicentrarchus labrax*, Linnaeus, 1758)
76 juveniles, Skalli and Robin (2004) showed that growth was positively correlated to dietary n-3
77 PUFA concentration. Consequently, global climate change has, along with direct effects,
78 indirect bottom-up effects on fish growth, notably via n-3 PUFA availability (Brett and
79 Groves, 1979; Jobling, 1996; Brett and Müller-Navarra, 2003).

80 Otoliths are calcified structures located in the inner ear of teleost fish that are characterized by
81 accretional growth and are metabolically inert (i.e. once deposited, otolith material is unlikely
82 to be resorbed or altered) (Casselman, 1987). Otolith shape is influenced by both abiotic (e.g.
83 temperature) and biotic (e.g. food availability) environmental parameters and depends on
84 individuals' genotype (Vignon and Morat, 2010). Since otolith growth is related to fish
85 growth (Mahé et al., 2017) and otolith shape co-varies with fish diet in the wild (Mille et al.
86 2016), global climate change may affect otolith morphogenesis and growth through its effects
87 on both temperature and n-3 PUFA availability. As stocks are characterized by different
88 environmental conditions and genetic makeup, associated variation in otolith growth, which
89 generates variation in otolith shape, may allow stock discrimination along species
90 distributional range (Cadrin et al., 2014). In fact, the otolith shape is commonly used as a tool

91 to identify species and to discriminate between fish stocks. Since Campana and Casselman's
92 seminal work (1993), many fishery scientists have used this approach for stock discrimination
93 studies in order to understand fish population dynamics and achieve reliable exploited fish
94 stock assessments for fishery management (Reiss et al., 2009). Therefore, it is crucial to
95 identify environmental variations that could influence otolith morphogenesis and growth. In
96 addition, the shape of otoliths could differ systematically (as opposed to randomly) between
97 right and left inner ears (e.g. side effect). A recent study on *Boops boops* showed that, for this
98 species, the amplitude of directional bilateral asymmetry (DA) varied geographically, at least
99 partly due to environmental conditions (Mahé et al., 2018).

100

101 **2. The present study investigates under controlled conditions the crossed effects of**
102 **temperature (15°C and 20°C) and dietary n-3 PUFA content (0.73% EPA+DHA**
103 **versus 1.65% EPA+DHA) on otolith morphogenesis and growth in European**
104 **seabass. We investigated how otolith morphological variability within**
105 **individuals, i.e. directional bilateral asymmetry, and between individuals is**
106 **affected by environmental drivers, more precisely water temperature and dietary**
107 **n-3 PUFA content, during the early life of seabass. This species is commercially**
108 **important for both fisheries and aquaculture in Europe, especially in the**
109 **Mediterranean Sea. The world aquaculture production of European seabass was**
110 **191.003 tons in 2016 (FAO FishStat data). The effects of temperature or dietary**
111 **n-3 PUFA concentration on seabass somatic growth have been well-studied for**
112 **aquaculture purposes (Person-Le Ruyet et al., 2004a, 2004b; Skalli and Robin,**
113 **2004), but their effects on otolith morphogenesis and growth are currently**
114 **unknown. *Materials and methods***

115 2.1. *Fish and rearing conditions*

116 Seabass adults were captured and then reared in captivity for three years, after which a batch
117 of 4 females and 10 males was used to obtain the eggs for this study. The experiment was
118 carried out in 2017 using 93-day-old juveniles that were offspring of adult European seabass
119 captured in winter 2013 in the Gulf of Morbihan (France). At 2 days post hatching (dph),
120 seabass larvae were divided among three conical tanks (230 L, 10µm filtered seawater, UV,

121 salinity 35‰, initial density 10 000 larvae tank⁻¹). Water temperature in the tanks was
122 progressively increased from 14°C to 20°C from 2 to 8 dph. From the mouth opening stage to
123 39 dph, larvae were fed with *Artemia*. At 40 dph, larvae were co-fed with *Artemia* and a
124 microparticulate diet (Marinstart, Le Gouessant, Lamballe, France) for four days, and then fed
125 exclusively with the microparticulate diet until 74 dph. Thereafter, juveniles were fed with
126 larger pellets for ornamental fish (EPA + DHA = 1.5%, Le Gouessant, Lamballe, France)
127 until the beginning of the experiment at 93 dph.

128 2.2. *Experimental design*

129 A two-level full-factorial experiment with temperature and dietary PUFA content as treatment
130 factors was conducted using 3 600 seabass from 93 to 204 dph (Fig. 1). 300 individuals were
131 reared in each of 12 indoor 500 L tanks supplied with filtered and aerated natural seawater.
132 Fish were reared at either 15 or 20°C. They were fed diets containing either only fish oil, a
133 reference n-3 PUFA diet (1.65% EPA+DHA, current level observed in wild fishmeal), or
134 mixed fish and vegetable oils (50% rapeseed oil and 50% fish oil), i.e. a low n-3 PUFA diet
135 (0.73% EPA+DHA corresponding to European seabass nutritional requirements, Gourtay,
136 2018). Experimental set-up was based on a full-factorial design, with 3 replicates (tanks) for
137 each experimental condition (i.e. temperature-food combination). Each day, feeding took
138 place for 7 h during daytime (08:00 to 15:00) using an automatic feeder (2 cm h⁻¹). For more
139 details on diets see Gourtay et al. (2018)

140 To follow-up the otolith growth and shape variation through time, fish were sampled 3 times
141 over the course of the experiment. At 93 dph, 30 individuals were sampled and then at the
142 other two samplings, 10 fish per tank (i.e. 30 fish per each experimental condition and each at
143 each time) were also sampled. To analyze the temperature effect on growth and shape of the
144 otolith, we used the “heat units approach” that has been developed since about 1730 (e.g.,
145 Réaumur, 1735), initially to quantify the thermal opportunity for the growth and the

146 development of plants by aggregating temperatures relevant to these processes. Designated by
 147 a number of expressions, including “thermal time”, “heat units”, “thermal units”, or “growth
 148 units”, thermal opportunity for growth is usually measured in “growing degree days” (*GDD*;
 149 Wang, 1960; Bonhomme 2000; Trudgill et al., 2005; Neuheimer and Taggart; 2007) that are
 150 calculated as follows:

$$GDD = \sum_{i=1}^n (T_i - T_{th}) \cdot \Delta d$$

151 where T_i represents the average temperature at day i , T_{th} is the threshold temperature below
 152 which growth is expected to cease and Δd is time (in days). In our study, T_{th} was set at 10°C
 153 to minimize the bias introduced by this factor (Charnov and Gillooly, 2003, Chezik et al.,
 154 2013). This threshold was chosen in accordance with Barnabé (1991) who showed that
 155 juvenile seabass growth ceased between 10 and 15°C. The *GDD* approach quantifies the
 156 thermal opportunity for growth by aggregating temperatures relevant to growth (Rittner,
 157 2009). It is thus more precise than the calendar time approach when describing growth
 158 (Neuheimer and Taggart, 2007). The first sampling occurred at 93 dph ($GDD=1\ 410\ ^\circ\text{C}\cdot\text{day}$).
 159 Then, the two other samplings were carried out depending on the rearing temperature, i.e. at
 160 129 and 176 dph for 20°C and at 141 and 204 dph for 15°C, which corresponded to
 161 $GDD=2\ 115\ ^\circ\text{C}\cdot\text{day}$ and $GDD=3\ 055\ ^\circ\text{C}\cdot\text{day}$ for the 2nd and 3rd sampling event, respectively,
 162 whatever rearing temperature (Fig. 1).

163 **2.3. Otolith shape analysis and measures**

164 After measuring the total length ($TL \pm 0.1\ \text{cm}$) of fish, their sagittal otoliths (left and right)
 165 were extracted from the cranial cavity and cleaned. In order to minimize distortion errors in
 166 the normalization process during image analysis, each otolith was placed on a microscope
 167 slide with the sulcus facing downward and the rostrum pointing left. The outline of each
 168 otolith was digitized using an image analysis system consisting of a high-resolution camera

169 (Hamamatsu Orca 3G) connected to an automated microscope using reflected light (Zeiss
 170 Axio Imager Z2). Each digitized image was analyzed using the image-analysis software
 171 TNPC (Digital processing for calcified structures, version 7). To compare the shapes of the
 172 left and right otoliths, mirror images of the right otoliths were used. Otolith shape was
 173 assessed by analyzing three types of descriptors: the otoliths' size parameters (Length: L_o ;
 174 Width: W_o ; Perimeter: P_o ; Area: A_o), shape indices (Table S1) and Elliptic Fourier
 175 Descriptors (EFDs; e.g. Lestrel, 2008). Size parameters are measurements directly related to
 176 the size of the otolith, unlike shape indices, which are dimensionless measurements and
 177 therefore independent of the size of the otolith. The shape indices measure otolith
 178 morphology similarity to ideal geometric shapes calculated using size parameters (Table S1):
 179 Roundness (Ord) and Form-factor (Off) quantify similarity to a circle, Ellipticity (Oel) to an
 180 ellipse, and Aspect-Ratio (Oar) and Rectangularity (Ore) to a rectangle. EFDs were obtained
 181 by using TNPC 7 software to perform Fourier analysis on binarized image. For each otolith,
 182 the first 99 elliptical Fourier harmonics (H) were extracted and normalized with respect to the
 183 first harmonic so as to be invariant to otolith size, rotation and starting point of contour
 184 description (Kuhl and Giardina, 1982). To determine the number of harmonics required to
 185 reconstruct the otolith outline, the cumulated Fourier Power (F) was calculated for each
 186 otolith k as a measure of the precision of contour reconstruction obtained with n_k harmonics
 187 (i.e., the proportion of variance in contour coordinates accounted for by the n_k harmonics):

$$F_{(n_k)} = \sum_{i=1}^{n_k} \frac{A_i^2 + B_i^2 + C_i^2 + D_i^2}{2}$$

188 where A_i , B_i , C_i and D_i are the coefficients of the i th harmonic. n_k was chosen for each
 189 otolith k in order to ensure that it was reconstructed with a precision of $F_{(n_k)} = 99.99\%$
 190 (Lestrel, 2008). The maximum number of harmonics $n = \max(n_k)$ across all otoliths was
 191 then used to reconstruct each individual otolith. Only the first 31 harmonics were necessary to

192 ensure reconstruction of each otolith shape with a precision of 99.99% and were thus used for
 193 further analyses.

194 The resulting matrix containing EFDs (as columns) for each otolith (as rows) was subjected to
 195 Principal Components Analysis (PCA) (Rohlf and Archie, 1984) and the 7 first Principal
 196 Components (PCs) were selected as otolith shape descriptors or shape matrix (S) according to
 197 the broken stick model (Legendre and Legendre, 1998), which in this case, corresponded to a
 198 threshold of 3.4% of the total variance explained (Borcard et al., 2011). In total, these 7 PC's
 199 explained 82.1% of the total variance in the EFD's .This procedure allowed us to decrease the
 200 number of variables used to describe otolith shape variability through EFDs while ensuring
 201 that the main sources of shape variation were kept and to avoid co-linearity between shape
 202 descriptors (Rohlf and Archie, 1984).

203 2.4 Statistical analyses

204 2.4.1 Directional bilateral asymmetry in otolith shape

205 To analyse the directional bilateral asymmetry (DA) between left and right otolith shape, the
 206 effect of inner ear side (Si) on otolith morphology O (described by either size parameters or
 207 shape indices or the shape matrix S) was tested using linear mixed-effects models (univariate
 208 models for size parameters, shape indices and multivariate models for shape matrix S) :

$$209 \quad O \sim \alpha_0 + \Delta\alpha_{0,in} + \alpha_1 Si + \alpha_2 GDD + \alpha_3 Si. GDD + \alpha_{4,PUFA} Si. GDD \quad (1)$$

210 where inner ear side (*Si*), growing degree day (*GDD*) and their interaction with dietary PUFA
 211 content (*PUFA*) are fixed effects and individual (*in*) is introduced as a random effect
 212 ($\Delta\alpha_{1,in}$) on the intercept to account for the fact that left and right otoliths' shapes are more
 213 likely to be similar when originating from the same individual. The *GDD* values were
 214 centered around the value at the beginning of the experiment (1 410 °C.day) so that the
 215 intercept of the model represents otolith morphology at that point in time.

216 The multivariate versions of model (1) applied to shape matrix S were fitted while accounting
 217 for variance heterogeneity between PCs (columns of S). Normality of the residuals and the
 218 random effects as well as homoscedasticity of the residuals were assessed by visual inspection
 219 of diagnostic plots. The models were fitted by maximizing the restricted log-likelihood. The
 220 significance of the fixed effects was tested by likelihood ratio tests between nested models
 221 while respecting marginality of the effects (type 2 tests; Fox and Weisberg, 2011) that are
 222 supposed to follow a χ^2 distribution under the null hypothesis. Their p-values were adjusted
 223 for Multiple Comparisons through the Bonferroni correction (McDonald, 2014).

224 To visualise shape differences between right and left otoliths, or DA amplitude, average left
 225 and right shapes were rebuilt based on EFDs averaged for each side. DA amplitude was then
 226 computed as the percentage of non-overlapping surface between the reconstructed right and
 227 left otolith average shapes relative to the total area they covered after superposition.

228 2.4.2 Temperature and food composition effects on otolith morphogenesis

229 To test Temperature (T) and food composition ($PUFA$) effects on otolith morphogenesis, i.e.
 230 the change with time in otolith shape O , four mixed-effects models (univariate or multivariate
 231 depending on shape descriptors) were fitted:

$$232 \quad O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})D + \alpha_2 D.T + \alpha_{3,PUFA}D + \alpha_{4,PUFA}D.T \quad (2)$$

$$233 \quad O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})GDD + \alpha_{2,PUFA}GDD \quad (3)$$

$$234 \quad O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})D + \alpha_2 D.GDD + \alpha_{3,PUFA}D + \alpha_{4,PUFA}D.GDD \quad (4)$$

$$235 \quad O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})GDD + \alpha_{2,PUFA}GDD + \alpha_3 GDD.T + \alpha_{4,PUFA}GDD.T \quad (5)$$

236 where sampling day (D), dietary PUFA content ($PUFA$, 0.73% / 1.65%), temperature (T ,
 237 15°C / 20°C), growing degree day (GDD), and their interaction are fixed effects. PUFA was a
 238 categorical variable in the 4 used models. In contrast, different options were applied to

239 account for the effect of temperature on otolith morphogenesis: either by considering
240 explicitly time D and temperature T (model 2) or by including a combined measure of thermal
241 time GDD (model 3) or by examining a combination of GDD and D (model 4) or GDD and T
242 (model 5) in case thermal time did not fully account for either real time effect or real
243 temperature effect. Random effects ($\Delta\alpha_{1,i}$) on D or GDD effect were used to account for
244 variability in otolith morphogenesis due to rearing tanks (i). These random effects affected
245 only the model slopes and not the intercepts because fish lived in the same tank until 93 dph,
246 i.e. the beginning of the full-factorial experiment at which $D = GDD = 0$.

247 We used the smallest Akaike's Information Criterion (AIC) to choose the most appropriate
248 model between the 4 options. Model fitting, diagnostics and significance tests of fixed effects
249 in the selected model were performed as described for DA analysis.

250 In a second step, individuals' total length TL was also included as a covariate in the selected
251 model that was re-fitted to estimate some potential confounding ontogenetic effects on otolith
252 shape, i.e. the fact that temperature or food composition could affect otolith shape indirectly
253 through their direct effect on fish body growth and the subsequent effect of fish body size on
254 otolith shape. Also, the potential sensibility of the results of these models to the threshold
255 temperature (Tth) used to compute GDDs was tested by varying the value of this parameter
256 from 0°C to 15°C by 1 degree increments and refitting the different models according to the
257 procedure described above.

258 Only the fixed part of the selected models is presented in the result section. Random effects,
259 which were included to account for variation due to tanks and thus to obtain robust estimates,
260 are presented in the Supplementary Material (Table S2). As for the DA analysis, to visualise
261 and to evaluate the amplitude of differences in otolith shape between pairs of groups
262 characterized by different rearing conditions, an average otolith shape by group was rebuilt

263 based on EFDs and the percentage of non-overlapping surface between reconstructed average
264 shapes of the two groups was calculated. Finally, the significance of shape difference between
265 all group pairs was tested using a mixed-effects model while correcting for multiple
266 comparisons using a Bonferroni procedure.

267 Statistical analyses were performed using the following packages in the statistical
268 environment R: ‘nlme’ (Pinheiro et al., 2016), ‘lme4’ (Bates et al., 2011), ‘Vegan’ (Oksanen
269 et al., 2013), ‘car’ (Fox et al., 2011) and ‘MASS’ (Venables and Ripley, 2002).

270 **3. Results**

271 **3.1 Directional bilateral asymmetry (DA) in otolith shape**

272 For all descriptors of otolith shape, the side value (i.e. the difference between the values
273 measured on the right otolith and on the left otolith), was always negative reflecting
274 measurements of the right otolith always lower than that of the left otolith (Table 1).
275 According to the aspect of otolith morphology considered, the mixed-effects models did not
276 show the same trend (Table 1). Regarding otolith size, represented by the four size parameters
277 (*Lo*, *Wo*, *Po* and *Ao*), DA was significant since the beginning of the experiment (column *Si*,
278 Table 1) but varied also according to experienced thermal opportunity (column *Si.GDD*,
279 Table 1) and this differently depending on dietary PUFA content (column *Si.GDD.PUFA*,
280 Table 1). Regarding otolith shape independent of size, represented by the other descriptors
281 (Shape indices and EFDs), some DA was again detected since the beginning of the
282 experiment (column *Si*, Table 1), except for rectangularity and the form-factor (lines *Ore* and
283 *Off*, respectively, Table 1). However, in contrast with otolith size, DA in otolith shape did
284 not vary with thermal opportunity (column *Si.GDD*, Table 1) nor with dietary PUFA content
285 (column *Si.GDD.PUFA*, Table 1) The DA amplitude was on average equal to 1.17%
286 throughout the experiment (Fig. 2). At 1410°C.day, the DA amplitude was 0.94% and

287 increased with thermal opportunity but the values were higher at 15°C (2115°C.day: 1.31%;
288 3055°C.day: 1.36%) than at 20°C (2115°C.day: 1.04%; 3055°C.day: 1.08%). Given that DA
289 in otolith shape was constant whatever thermal opportunity (GDD) and dietary PUFA content,
290 only right otoliths were used in the following analyses.

291 *3.2 Temperature and food composition effects on otolith morphogenesis*

292 Among the four tested mixed-effects models, model (5) was always the best one according to
293 the AIC whatever the response variable (Table 2). This model included *GDD*, *PUFA*, *T* and
294 their interactions as fixed effects and applied similarly to fish somatic growth (i.e. fish total
295 length as response variable), otolith growth (i.e. size parameters as response variables) and
296 otolith morphogenesis (shape indices and matrix *S* as response variable). This suggests that
297 thermal opportunity (*GDD*) was a better descriptor than the separate effects of time (*D*) and
298 temperature (*T*) as represented in model 2. Thermal opportunity alone (model 3) was enough
299 to account for time effect but it was not to account for the full temperature effect, so that
300 interactions with time (*D*) were not necessary (model 4) but those with temperature (*T*) were
301 (model 5).

302 Despite better support for model 5 by AIC whatever the response variable, significance of the
303 different fixed effects varied across explained variables (Table 3). Fish growth (*TL* as
304 response variable) depended significantly on *GDD* but also on its interaction with *T*. Otolith
305 growth (size parameters as response variables, Table 3) depended significantly only on *GDD*
306 (response variables: *Lo*, *Po*, *Wo*) except for growth in otolith area (*Ao*) for which the
307 interaction with *T* was also significant. For otolith morphogenesis (shape indices and matrix *S*
308 as response variables, Table 3), results were more balanced with some indices (*Oel*, *Ord* and
309 *Oar*) indicating a significant effect of *GDD* only and others (*Ore*, *Off* and matrix *S*) also of
310 the interaction with *T*. It looked like the more complex was the index used to describe otolith
311 shape, the more likely to observe a significant interaction between *GDD* and *T*. In contrast,

312 the concentration of PUFA in diet had no significant effect on fish and otolith growth nor on
313 otolith morphogenesis (Table 3).

314 To test the effect of fish body size on the otolith shape, individual total length TL was
315 included as a covariate in model 5. There was no significant ontogenetic effect on the otolith
316 shape. . Moreover, the effect of the temperature threshold (Tth) was also tested in model 5.
317 While modifying the value of Tth from 0°C to 15°C by 1-degree increment, no changes
318 occurred in the significance of explanatory variables (Table S3).

319 The evolution of normalized otolith shape during the experiment showed that there were two
320 main changes which were related to the dorso-ventral axis and the area between the rostrum
321 and anti-rostrum (Fig. 3). The otolith shape was modified by a decrease of width/length ratio
322 and of the distance between the rostrum and the anti-rostrum as GDD and T increased. When
323 comparing average shapes observed for each condition and each sampling time to the first one
324 at 93 dph (Fig. 3), we observed that differences between temperatures at similar GDD were
325 only concerning the amplitude of the modification relative to the initial otolith shape (in other
326 terms, the speed of otolith morphogenesis) and not the direction of the modification (in other
327 terms the trajectory of otolith morphogenesis). At 20°C, otolith shape changed faster than at
328 15°C. This was evidenced by the percentage of non-overlapping surface between average
329 otolith shape at 93 dph and that at various points in “thermal time” later which was always
330 significantly higher at 20°C than at 15°C at the same “thermal time” (GDD ; Table 4). In
331 contrast, the percentage of non-overlapping surface between average otolith shapes for
332 different dietary PUFA concentrations at the same GDD and T values was always lower than
333 2 % and was never significant (Table 4).

334 4. Discussion

335 4.1 Directional bilateral asymmetry in seabass otolith

336 In the present experiment, we found evidence of DA in otolith size reflecting measurements
337 of size parameters (length, width, area and perimeter) from the right otolith that are always
338 lower than that of the left otolith. The size of otoliths differed systematically (as opposed to
339 randomly) between right and left inner ears (e.g. side effect). The amplitude of DA varied
340 with GDD and n-3 PUFA concentration in diet. This indicates that the amplitude of the size
341 difference between left and right otoliths depends on thermal regime and diet quality during
342 the early-juvenile phase. Such directional bilateral asymmetry (DA) in shape (as opposed to
343 fluctuating asymmetry) is more important for flatfish than for roundfish because
344 metamorphosis causes unique asymmetric body shape and a lateralized behavior adapted to a
345 bottom-living lifestyle (Okada et al., 2001, Mille et al., 2015). However, significant DA has
346 been observed for roundfish species such as *Liza ramada* (Rebaya et al., 2017), *Diplodus*
347 *annularis* (Trojette et al., 2015), *Diplodus puntazzo* (Bostanci et al., 2016), *Clupea harengus*
348 (Bird et al., 1986), *Scomberomorus niphonius* (Zhang et al., 2016) and *Merlangius merlangus*
349 (Mille et al., 2015). A recent study on *Boops boops* showed that, for this species, the
350 amplitude of DA varied geographically, at least partly due to environmental conditions (Mahé
351 et al., 2018). The variability of otolith DA in *Boops boops* at least could be related to
352 population geographical structuration and thus could be a phenotypically plastic response to
353 environmental drivers, such as temperature, current patterns and food availability, and/or
354 result from genetic differentiation between geographical locations. Moreover, the ontogenetic
355 trajectory of otolith shape is affected by environmental conditions during early life stages
356 (Vignon, 2018), thus DA and its amplitude could vary accordingly. Although we found a
357 significant DA of the otolith size, in size-normalized otolith shape, the DA of the otolith shape
358 was not significant, and it was not affected by the environmental temperature nor food

359 quality, suggesting that these two factors do not affect DA in otolith shape in juvenile seabass.
360 This study suggests that for seabass, considering a similar trajectory of otolith morphogenesis
361 between left and right sides, otolith growth rate is different which could reveal a functional
362 adaptation with lateralization of hearing functions.

363

364

365 ***4.2 Effect of dietary n-3 PUFA concentration on otolith morphogenesis***

366 Water temperature influences essential n-3 PUFA content in zooplankton and consequently its
367 availability to species at higher trophic levels such as fish (Gladyshev et al., 2011), which
368 may impair their growth. Skalli and Robin (2004) showed that, indeed, below a minimal
369 threshold (0.70%), dietary concentration of n-3 PUFA was positively correlated to mass
370 growth in European sea bass juveniles. In this study, our results showed that the effect of
371 dietary n-3 PUFA content on otolith morphogenesis was not linked to environmental
372 conditions, especially temperature. Using juvenile sea bass from the same experiment as the
373 present paper, Gourtay et al. (2018) found lower body growth and a significant decrease in n-
374 3 PUFA content in muscle tissues when fish oil was partly replaced by vegetable oil. The
375 dietary n-3 PUFA content also affected organ mass allometry with total mass for the heart and
376 the gastrointestinal system being smaller when temperature was high (20°C), whereas those of
377 liver and mesenteric fat were affected at lower temperatures (15°C) (Gourtay et al., 2018).
378 This suggests that environmental conditions, especially temperature, could also modify the n-
379 3 PUFA metabolism pathways in fish and thus, modulate several physiological functions, as
380 found in other species (Farkas et al., 1980; Olsen and Skjervold, 1995). Besides somatic
381 growth, Benitez-Santana et al. (2007) showed that the dietary n-3 PUFA could influence
382 swimming activities in gilthead seabream (*Sparus aurata*), which may be related to effects on
383 the sensory system. Dietary n-3 PUFA are clearly necessary for brain and retina development

384 during the first life stages of egg, larvae and juveniles (Bell and Dick, 1991; Furuita et al.,
385 1998). For example, it has been shown that an elevation of dietary DHA and EPA content
386 increases eye diameter of gilthead seabream larvae (Izquierdo et al., 2000). Similarly, dietary
387 n-3 PUFA could have a potential effect on the fish audio-vestibular system development and
388 especially on otolith biomineralisation, which relies on a minor organic fraction representing
389 0.2 to 10% of their total material (Degens et al., 1969). One hypothesis was that diet
390 composition may affect the synthesis of endolymph organic constituents by the saccular
391 epithelium, notably saccular endolymph proteins that are precursors of the otolith organic
392 matrix, and this probably conditions the biomineralisation process and the morphogenesis of
393 otoliths during the first life stages of fish. More specifically, n-3 PUFA, through their effect
394 on membrane viscosity, could affect the transport of the organic precursors in the endolymph
395 by the saccular epithelium and thus affect otolith biomineralisation. However, no evidence for
396 an effect of dietary n-3 PUFA content on the morphogenesis of otoliths in sea bass juveniles
397 was observed in the present study. Therefore, these results do not exclude a potential effect of
398 the dietary n-3 PUFA content on otolith morphogenesis below the minimal threshold.

399 ***4.3. Growing Degree Day is good proxy to represent temperature effect on otolith*** 400 ***morphogenesis***

401 The thermal opportunity concept, often measured by growing degree days (GDD), has been
402 widely used for linking temperature and climate effects on the growth of plants and field
403 crops (Bonhomme, 2000; Trudgill et al., 2005). In fisheries sciences, this approach is still
404 underused to analyse fish development and growth. An extensive review of the literature
405 (Web of Science) showed that since 2000, 5 to 8 papers per year have used this concept,
406 which represents only 3 to 5% of temperature-related growth studies in fish. More
407 specifically, we did not find any studies on otolith morphogenesis that used GDD to follow
408 the ontogeny of otolith shape. We found that the best model to describe otolith morphogenesis
409 (whatever the descriptor of otolith size and shape), always included GDD and not calendar

410 time (D). This suggests that thermal opportunity or thermal time is more relevant to otolith
411 growth and morphogenesis than calendar time.

412 Fish growth and temperature are always linked (Weatherley and Gill, 1987) because, over a
413 mid-range of temperatures, somatic growth depends on metabolic enzyme activity, which is
414 necessarily temperature dependent (Jobling, 1994). The GDD approach accounts for this
415 relationship but assumes that growth is only a function of temperature and that this function is
416 linear (Legg, 1998). However, there are other biotic or abiotic factors (e.g. population density,
417 genetic make-up, food availability or composition, oxygen availability which may modulate
418 the effect of temperature on fish growth. Under the assumption that temperature is the main
419 contributor to growth, GDD can be a good descriptor of temperature-dependent processes that
420 have been proven to be more relevant than calendar time and temperature value for growth in
421 some fish species (Uphoff et al., 2013). Notably, Neuheimer and Taggart (2007) showed that
422 variation in length-at-age among 17 stocks of North Atlantic cod (*Gadus morhua*) was
423 significantly correlated with GDD and not with calendar time. Our study shows similar trend
424 with seabass growth and otolith morphogenesis. However, the use of GDD required testing
425 potential effects of T values. Moreover, the effect of total length was tested in the models.
426 Similarly to Chezik et al. (2013), our study showed that the value of T (from 0°C to 15°C in 1
427 degree increments) did not significantly contribute to the model for fish length or otolith
428 shape. The value of T can bias the analysis only among populations, not within one population
429 (Chezik et al., 2013). In the same way, the models including GDD did not show any
430 interaction or influence with the fish total length covariate. Consequently, there was no direct
431 size-dependent effect on the otolith shape. Thus using GDD seems to be very appropriate to
432 test the effect of temperature on otolith morphogenesis.

4.4 Effect of water temperature on the somatic growth and otolith shape

433 The temperature effect, in this study, was explained by only GDD for several shape
434 descriptors (e.g. Lo, Po, Wo, Oel, Ord and Oar), and by GDD together with the GDD-T
435 relationship for other shape descriptors (e.g. Ao, Oci, Ore, Off and PCs). This difference
436 between the shape descriptors is explained by the level of details of the contour that is
437 integrated in the descriptor. Many of the commonly used descriptors (e.g. size parameters,
438 shape indices and EFDs) for otolith shape are correlated (Table S4). Hence, the number of
439 used descriptors could be decreased. In seabass, the otolith morphogenesis takes place in two
440 stages, firstly, a decrease of the width/length ratio and secondly, a modification on the surface
441 area between the rostrum and the anti-rostrum. In cod (*Gadus morhua*), the otolith shape
442 increases in crenulations and the number of lobes (Hüssy, 2008). Several shape descriptors
443 (length, perimeter, width, ellipticity, roundness and aspect ratio) are not able to describe this
444 type of development. Conversely, the weight or the volume of the otolith could be good
445 proxies to characterize otolith shape in three dimensions. Moreover, the reconstruction of
446 individual evolution of otolith shape could be used to better understand the biomineralisation
447 process and consequently the resulting otolith shape (Fablet et al., 2009).

449 Temperature affects fish growth through direct effects (e.g. metabolism) and indirect effects
450 (e.g. relationship with biotic factors such as availability of food) (Uphoff et al., 2013). In a
451 controlled experimental environment, we minimized indirect food effects. In the same way,
452 we tried to reduce the genetic background effect by using 4 females and 10 males to produce
453 the individuals used for this study. However, we cannot exclude individual genetic effects that
454 may have influenced the effect of temperature, since genetics directly affect the growth of
455 poikilotherms by influencing enzymatic activity and the stability of proteins configuration
456 and, therefore, the metabolic rates (Higley et al., 1986; Clarke and Johnston, 1999; Pauly,
457 2010). Fish exhibit higher growth rates when their metabolic activity rises with increasing
458 temperature (in mid-range temperatures, Clarke and Johnston, 1999). Our results on seabass

459 corroborated this observation and also showed the same trend for otolith growth. A previous
460 experimental study with zooplanktivorous age-0 walleye (*Sander vitreus*) had shown that food
461 consumption rates could also increase with increasing temperatures (Johnston and Mathias,
462 1994). Another recent study using carbon isotopic ratios with juvenile Australasian snapper
463 (*Chrysophrys auratus*) reared in the laboratory showed that the $\delta^{13}\text{C}$ in otoliths was not linked
464 to the dietary regime but rather temperature because of an overall increase of metabolic rates
465 in all fish tissues (Martino et al., 2019). Our study, which indicated that the effect of
466 temperature on otolith shape was the sum of GDD value and the interaction between GDD
467 and mean temperature, shows that the effect of temperature is to accelerate otolith
468 morphogenesis and to modify the evolution trajectory because at fixed GDD values, for any
469 two given temperatures, the otolith shapes are different.

470

471 **Conclusion**

472 Our results indicate that otolith shape is more sensitive to the environmental temperature than
473 to food quality (i.e. EPA and DHA contents) and, as such, can be an effective tool for
474 identifying fish stocks living in different thermal environments. Growing Degree Day (GDD)
475 is a very efficient tool to analyze the temperature effect on otolith growth. However, otolith
476 shape is also influenced by the relationship between GDD and water temperature. In fact,
477 otolith shape is significantly influenced by the cumulative sum of GDD, but the evolution
478 trajectory of the otolith morphogenesis is positively correlated with the temperature of the
479 environment. In fact,

480

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741 Table 1 : Statistical testing of directional bilateral asymmetry in otolith size and shape. p-
 742 values obtained in the mixed-effects models (1) for the effects of inner head side effect (*Si*)
 743 and their interactions with growing degree days (*GDD*) and food composition (*PUFA*)
 744 (Bonferroni corrected p-values for Multiple Comparisons: p-values < 0.0045: grey cases).
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Type of data	Response variable	Si value	Si	GDD	Si.GDD	Si.GDD.PUFA
Size parameters	Length : Lo	-0.691	< 0.001	< 0.001	< 0.001	< 0.001
	Perimeter : Po	-0.359	< 0.001	< 0.001	0.001	< 0.001
	Area: Ao	-0.700	< 0.001	< 0.001	< 0.001	< 0.001
	Width : Wo	-0.698	< 0.001	< 0.001	0.008	< 0.001
Shape indices	Rectangularity : Ore	-0.513	0.104	< 0.001	0.464	0.274
	Ellipticity : Oel	-0.475	< 0.001	< 0.001	0.934	0.600
	Roundness : Ord	-0.410	< 0.001	< 0.001	0.963	0.121
	Aspect ratio : Oar	-0.450	< 0.001	< 0.001	0.784	0.382
	Form-factor : Off	-0.316	0.959	< 0.001	0.797	0.444
EFDs	matrix S		0.046	0.040	0.999	0.344

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Table 2: Δ AICfor the 4 alternative mixed-effects models (columns) fitted to the different
 response variables (lines) describing fish total length (TL) and otolith morphology (size
 parameters, shape indices and matrix S).

Response variable	model 2 : <i>D+D:T+D:PUFA+ D:T:PUFA+i</i>	model 3 : <i>GDD+GDD:PUFA+i</i>	model 4 : <i>D+D:GDD+D:PUFA+D:G DD:PUFA+i</i>	model 5 : <i>GDD+GDD:PUFA+GDD :T+GDD:PUFA:T+i</i>
Fish growth	94	332	293	0
Size	28	168	144	0

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parameters	<i>Po</i>	39	161	140	0
	<i>Ao</i>	29	206	178	0
	<i>Wo</i>	27	190	158	0

Shape indices	<i>Ore</i>	94	332	304	0
	<i>Oel</i>	22	18	19	0
	<i>Ord</i>	18	1	4	0
	<i>Oar</i>	26	3	4	0
	<i>Off</i>	42	33	5	0

EFDs	<i>PCs</i>	3861	308	4087	0
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Table 3: Results of mixed-effects models testing for the effects of food composition (*PUFA*, 0.73%/1.65%), experimental temperature (*T*, 15°C/20°C), growing degree day (*GDD*), and their interaction for each response variable (Adjusted P-values for Multiple Comparisons: p-values < 0.0045: **).

Type of data	Response variable	Explanatory variable	χ^2	df	ρ -value
Fish growth	TL	GDD	3404.4205	1	< 0.001 **
		GDD:PUFA	1.9803	1	0.159
		GDD:T	42.6184	1	< 0.001 **
		GDD:PUFA:T	1.0344	1	0.309
Size parameters	Lo	GDD	650.4437	1	< 0.001 **
		GDD:PUFA	0.0080	1	0.928
		GDD:T	0.0986	1	0.753
		GDD:PUFA:T	0.0022	1	0.963
	Po	GDD	727.4510	1	< 0.001 **
		GDD:PUFA	0.0233	1	0.879
		GDD:T	0.0579	1	0.810
		GDD:PUFA:T	0.0210	1	0.885
	Ao	GDD	4291.9324	1	< 0.001 **
		GDD:PUFA	2.9141	1	0.088
		GDD:T	28.3203	1	< 0.001 **
		GDD:PUFA:T	1.1427	1	0.285
	Wo	GDD	322.1920	1	< 0.001 **
		GDD:PUFA	0.0076	1	0.930
		GDD:T	0.0217	1	0.883
		GDD:PUFA:T	0.0705	1	0.791
Shape indices	Ore	GDD	95.8393	1	< 0.001 **
		GDD:PUFA	0.0974	1	0.7550
		GDD:T	9.3282	1	0.002 **

		GDD:PUFA:T	0.2599	1	0.610
	Oel	GDD	527.4	1	< 0.001 **
		GDD:PUFA	2.301	1	0.129
		GDD:T	3.6395	1	0.056
		GDD:PUFA:T	0.0583	1	0.809
	Ord	GDD	581.3443	1	< 0.001 **
		GDD:PUFA	2.4954	1	0.114
		GDD:T	2.4173	1	0.120
		GDD:PUFA:T	0.0263	1	0.871
	Oar	GDD	549.2033	1	< 0.001 **
		GDD:PUFA	2.1935	1	0.139
		GDD:T	2.6449	1	0.104
		GDD:PUFA:T	0.0314	1	0.859
	Off	GDD	196.6761	1	< 0.001 **
		GDD:PUFA	2.6622	1	0.103
		GDD:T	74.3587	1	< 0.001 **
		GDD:PUFA:T	0.3677	1	0.544
EFDs	matrix S	GDD:PUFA	4.1173	7	0.766
		GDD:T	71.5240	7	< 0.001 **
		GDD:PUFA:T	7.3534	7	0.393

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Table 4: Average percentage of non-overlapping surface between reconstructed average otolith shapes for pairs of rearing conditions (**: p-value<0.05).

		141 0 °C.d ay	2155 °C.day				3055 °C.day			
non-overlap (%)		D93	D129 1.85% 20°C	D129 0.85% 20°C	D141 1.85% 15°C	D141 0.85% 15°C	D176 1.85% 20°C	D176 0.85% 20°C	D204 1.85% 15°C	D204 0.85% 15°C
1410 °C.day	D93									
2155 °C.day	D129_1.85% _20°C	7.82 **								
	D129_0.85% _20°C	8.36 **	1.85							
	D141_1.85% _15°C	4.35 **	5.14 **	5.93 **						
	D141_0.85% _15°C	3.00 **	6.61 **	7.34 **	1.89					
3055 °C.day	D176_1.85% _20°C	12.04 **	5.47 **	4.55 **	9.96 **	9.85 **				
	D176_0.85% _20°C	11.10 **	4.51 **	3.67 **	8.90 **	8.75 **	1.64			

D204_1.85% _15°C	7.59 **	2.74 **	2.56 **	4.52 **	4.64 **	6.10 **	4.25 **		
D204_0.85% _15°C	6.91 **	3.19 **	3.32 **	3.76 **	3.85 **	6.85 **	5.11 **	1.64	

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772 Figure 1: Main steps of the experimental design for seabass with 5 samplings at 93, 129, 141,
 773 176 and 204 dph. Each group (G) was composed of 3 replicates. To test the temperature
 774 effect, 15 °C (blue) and 20 °C (red) were used and for concentrations of PUFA, 1.85 (dotted)
 775 and 0.85 (striated) were used.

776 Figure 2: Difference between *left (black dotted line) and right (grey line) average otolith*
 777 *shape.*

778 Figure 3: Evolution of otolith normalized shape during the experiment: D-93 (1410°C day;
 779 black line), D-129 (2155°C day; 20°C; red dashed line), D-146 (2155°C day; 15°C; blue dashed
 780 line), D-176 (3055°C day; 20°C; red line) and D-204 (3055°C day; 15°C; blue line).

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