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

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Abstract :

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

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

Graphical abstract
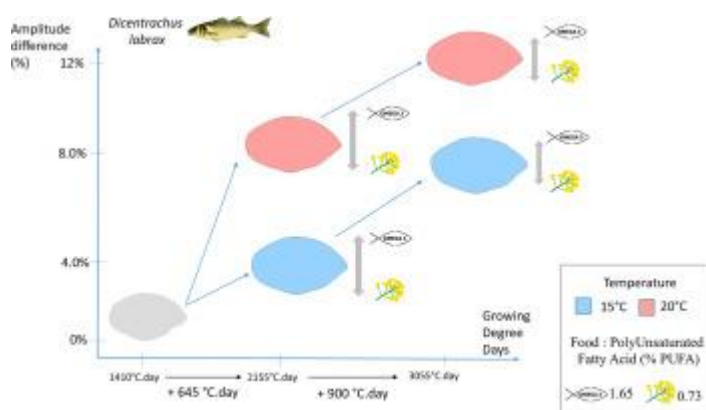
Highlights

► No directional bilateral asymmetry found between left and right otoliths during early life stages of seabass ► Growing Degree Days is best descriptor to follow the temperature effect on otolith shape ► Otolith shape is more sensitive to temperature than to food quality (dietary polyunsaturated fatty acid content)

Keywords : n-3 polyunsaturated fatty acids, Otolith shape, Temperature effect, Elliptic Fourier descriptors, Multivariate mixed-effects models, Directional asymmetry

1. Introduction

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

Global climate change also induces ocean acidification and deoxygenation episodes (Diaz, 2001; Caldeira and Wickett, 2003; Pörtner and Knust, 2007; Feely et al., 2008; Rabalais et al.,

2009). These environmental changes could reduce and modify the base of marine food webs, notably phytoplankton species assemblages, with potential negative consequences on the production of omega-3 polyunsaturated fatty acids (n-3 PUFA) (Ackman and Tocher 1968; Thompson et al., 1992; Renaud et al. 2002; Guschina and Harwood 2006; Capuzzo et al., 2017). Fatty acids are the primary constituents of polar and neutral lipids, specifically phospholipids and triacylglycerols, respectively. N-3 PUFA, particularly eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), contained in phospholipids (Henderson and Tocher, 1987), are exclusively synthesized by phytoplankton species. There is a negative correlation between temperature and the production of n-3 PUFA by phytoplankton. n-3 PUFA have a critical role as the primary building blocks of cell membranes as components of phospholipids, according to the concept of homeoviscous adaptation (i.e. adjustment of the n-3 PUFA to ensure the viscosity and membrane exchanges necessary for the proper functioning of the organism; Ernst et al., 2016). N-3 PUFA are essential fatty acids (EFA) that animals, including fish, must ingest as they are almost exclusively produced by phytoplankton and can rarely be synthesized *de novo* by the animals themselves (Brett and Müller-Navarra, 2003). A climate-change-induced reduction in dietary PUFA availability could thus impact fish physiology by changing cell membrane fluidity, the production of eicosanoids and main physiological processes (growth, respiration and reproductive rates) with potential consequences on fish recruitment (Brett and Müller-Navarra, 2003). For example, in European seabass (*Dicentrarchus labrax*, Linnaeus, 1758) juveniles, Skalli and Robin (2004) showed that growth was positively correlated to dietary n-3 PUFA concentration. Consequently, global climate change has, along with direct effects, indirect bottom-up effects on fish growth, notably via n-3 PUFA availability (Brett and Groves, 1979; Jobling, 1996; Brett and Müller-Navarra, 2003).

82   Otoliths are calcified structures located in the inner ear of teleost fish that are characterized by

83   accretional growth and are metabolically inert (i.e. once deposited, otolith material is unlikely

84   to be resorbed or altered) (Casselman, 1987).Otolith shape is influenced by both abiotic (e.g.

85   temperature) and biotic (e.g. food availability) environmental parameters and depends on

86   individuals' genotype (Vignon and Morat, 2010). Since otolith growth is related to fish

87   growth (Mahé et al., 2017) and otolith shape co-varies with fish diet in the wild (Mille et al.

88   2016), global climate change may affect otolith morphogenesis and growth through its effects

89   on both temperature and n-3 PUFA availability. As stocks are characterized by different

90   environmental conditions and genetic makeup, associated variation in otolith growth, which

91   generates variation in otolith shape, may allow stock discrimination along species

92   distributional range (Cadrin et al., 2014). In fact, the otolith shape is commonly used as a tool

93   to identify species and to discriminate between fish stocks. Since Campana and Casselman's

94   seminal work (1993), many fishery scientists have used this approach for stock discrimination

95   studies in order to understand fish population dynamics and achieve reliable exploited fish

96   stock assessments for fishery management (Reiss et al., 2009). Therefore, it is crucial to

97   identify environmental variations that could influence otolith morphogenesis and growth. In

98   addition, the shape of otoliths could differ systematically (as opposed to randomly) between

99   right and left inner ears (e.g. side effect). A recent study on *Boops boops* showed that, for this

100   species, the amplitude of directional bilateral asymmetry (DA) varied geographically, at least

101   partly due to environmental conditions (Mahé et al., 2018).

102   The present study investigates under controlled conditions the crossed effects of temperature (15°C

103   and 20°C) and dietary n-3 PUFA content (0.73% EPA+DHA versus 1.65% EPA+DHA) on otolith

104   morphogenesis and growth in European seabass. We investigated how otolith morphological

105   variability within individuals, i.e. directional bilateral asymmetry, and between individuals is affected

106   by environmental drivers, more precisely water temperature and dietary n-3 PUFA content, during

107   the early life of seabass. This species is commercially important for both fisheries and aquaculture in

108   Europe, especially in the Mediterranean Sea. The world aquaculture production of European seabass

109   was 191.003 tons in 2016 (FAO FishStat data). The effects of temperature or dietary n-3 PUFA

110   concentration on seabass somatic growth have been well-studied for aquaculture purposes (Person-

111   Le Ruyet et al., 2004a, 2004b; Skalli and Robin, 2004), but their effects on otolith morphogenesis and

112   growth are currently unknown.

113   ### 1. Materials and methods

114   ### 1.1. Fish and rearing conditions

115   Seabass adults were captured and then reared in captivity for three years, after which a batch

116   of 4 females and 10 males was used to obtain the eggs for this study. The experiment was

117   carried out in 2017 using 93-day-old juveniles that were offspring of adult European seabass

118   captured in winter 2013 in the Gulf of Morbihan (France). At 2 days post hatching (dph),

119   seabass larvae were divided among three conical tanks (230 L, 10μm filtered seawater, UV,

120   salinity 35‰, initial density 10 000 larvae tank^{-1}). Water temperature in the tanks was

121   progressively increased from 14°C to 20°C from 2 to 8 dph. From the mouth opening stage to

122   39 dph, larvae were fed with *Artemia*. At 40 dph, larvae were co-fed with *Artemia* and a

123   microparticulate diet (Marinstart, Le Gouessant, Lamballe, France) for four days, and then fed

124   exclusively with the microparticulate diet until 74 dph. Thereafter, juveniles were fed with

125   larger pellets for ornamental fish (EPA + DHA = 1.5%, Le Gouessant, Lamballe, France)

126   until the beginning of the experiment at 93 dph.

127   ### 1.2. Experimental design

128   A two-level full-factorial experiment with temperature and dietary PUFA content as treatment

129   factors was conducted using 3 600 seabass from 93 to 204 dph (Fig. 1). 300 individuals were

130   reared in each of 12 indoor 500 L tanks supplied with filtered and aerated natural seawater.

131   Fish were reared at either 15 or 20°C. They were fed diets containing either only fish oil, a

132   reference n-3 PUFA diet (1.65% EPA+DHA, current level observed in wild fishmeal), or

133   mixed fish and vegetable oils (50% rapeseed oil and 50% fish oil), i.e. a low n-3 PUFA diet

134   (0.73% EPA+DHA corresponding to European seabass nutritional requirements, Gourtay,

135   2018). Experimental set-up was based on a full-factorial design, with 3 replicates (tanks) for

136   each experimental condition (i.e. temperature-food combination). Each day, feeding took

137   place for 7 h during daytime (08:00 to 15:00) using an automatic feeder (2 cm h^{-1}). For more

138   details on diets see Gourtay et al. (2018)

139   To follow-up the otolith growth and shape variation through time, fish were sampled 3 times

140   over the course of the experiment. At 93 dph, 30 individuals were sampled and then at the

141   other two samplings, 10 fish per tank (i.e. 30 fish per each experimental condition and each at

142   each time) were also sampled. To analyze the temperature effect on growth and shape of the

143   otolith, we used the "heat units approach" that has been developed since about 1730 (e.g.,

144   Réaumur, 1735), initially to quantify the thermal opportunity for the growth and the

145   development of plants by aggregating temperatures relevant to these processes. Designated by

146   a number of expressions, including "thermal time", "heat units", "thermal units", or "growth

147   units", thermal opportunity for growth is usually measured in "growing degree days" (GDD;

148   Wang, 1960; Bonhomme 2000; Trudgill et al., 2005; Neuheimer and Taggart; 2007) that are

149   calculated as follows:

$$GDD = \sum_{i=1}^{n}(Ti - Tth).\Delta d$$

150   where Ti represents the average temperature at day i, Tth is the threshold temperature below

151   which growth is expected to cease and Δd is time (in days). In our study, Tth was set at 10°C

152   to minimize the bias introduced by this factor (Charnov and Gillooly, 2003, Chezik et al.,

153   2013). This threshold was chosen in accordance with Barnabé (1991) who showed that

154   juvenile seabass growth ceased between 10 and 15°C. The GDD approach quantifies the

155 thermal opportunity for growth by aggregating temperatures relevant to growth (Rittner,

156 2009). It is thus more precise than the calendar time approach when describing growth

157 (Neuheimer and Taggart, 2007). The first sampling occurred at 93 dph (GDD=1 410 °C.day).

158 Then, the two other samplings were carried out depending on the rearing temperature, i.e. at

159 129 and 176 dph for 20°C and at 141 and 204 dph for 15°C, which corresponded to

160 GDD=2 115°C.day and GDD=3 055°C.day for the 2nd and 3rd sampling event, respectively,

161 whatever rearing temperature (Fig. 1).

162 ### 1.3. Otolith shape analysis and measures

163 After measuring the total length (TL± 0.1 cm) of fish, their sagittal otoliths (left and right)

164 were extracted from the cranial cavity and cleaned. In order to minimize distortion errors in

165 the normalization process during image analysis, each otolith was placed on a microscope

166 slide with the sulcus facing downward and the rostrum pointing left. The outline of each

167 otolith was digitized using an image analysis system consisting of a high-resolution camera

168 (Hamamatsu Orca 3G) connected to an automated microscope using reflected light (Zeiss

169 Axio Imager Z2). Each digitized image was analyzed using the image-analysis software

170 TNPC (Digital processing for calcified structures, version 7). To compare the shapes of the

171 left and right otoliths, mirror images of the right otoliths were used. Otolith shape was

172 assessed by analyzing three types of descriptors: the otoliths' size parameters (Length: Lo;

173 Width: Wo; Perimeter: Po; Area: Ao;), shape indices (Table S1) and Elliptic Fourier

174 Descriptors (EFDs; e.g. Lestrel, 2008). Size parameters are measurements directly related to

175 the size of the otolith, unlike shape indices, which are dimensionless measurements and

176 therefore independent of the size of the otolith. The shape indices measure otolith

177 morphology similarity to ideal geometric shapes calculated using size parameters (Table S1):

178 Roundness (Ord) and Form-factor (Off) quantify similarity to a circle, Ellipticity (Oel) to an

179 ellipse, and Apect-Ratio (Oar) and Rectangularity (Ore) to a rectangle. EFDs were obtained

180   by using TNPC 7 software to perform Fourier analysis on binarized image. For each otolith,

181   the first 99 elliptical Fourier harmonics (H) were extracted and normalized with respect to the

182   first harmonic so as to be invariant to otolith size, rotation and starting point of contour

183   description (Kuhl and Giardina, 1982). To determine the number of harmonics required to

184   reconstruct the otolith outline, the cumulated Fourier Power (F) was calculated for each

185   otolith k as a measure of the precision of contour reconstruction obtained with n_k harmonics

186   (i.e., the proportion of variance in contour coordinates accounted for by the n_k harmonics):

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

187   where A_i, B_i, C_i and D_i are the coefficients of the ith harmonic. n_k was chosen for each

188   otolith k in order to ensure that it was reconstructed with a precision of $F_{(n_k)} = 99.99\%$

189   (Lestrel, 2008). The maximum number of harmonics $n = \max(n_k)$ across all otoliths was

190   then used to reconstruct each individual otolith. Only the first 31 harmonics were necessary to

191   ensure reconstruction of each otolith shape with a precision of 99.99% and were thus used for

192   further analyses.

193   The resulting matrix containing EFDs (as columns) for each otolith (as rows) was subjected to

194   Principal Components Analysis (PCA) (Rohlf and Archie, 1984) and the 7 first Principal

195   Components (PCs) were selected as otolith shape descriptors or shape matrix (S) according to

196   the broken stick model (Legendre and Legendre, 1998), which in this case, corresponded to a

197   threshold of 3.4% of the total variance explained (Borcard et al., 2011). In total, these 7 PC's

198   explained 82.1% of the total variance in the EFD's .This procedure allowed us to decrease the

199   number of variables used to describe otolith shape variability through EFDs while ensuring

200   that the main sources of shape variation were kept and to avoid co-linearity between shape

201   descriptors (Rohlf and Archie, 1984).

202   *2.4   Statistical analyses*

203   2.4.1 *Directional bilateral asymmetry* in otolith shape

204   To analyse the directional bilateral asymmetry (DA) between left and right otolith shape, the

205   effect of inner ear side (Si) on otolith morphology O (described by either size parameters or

206   shape indices or the shape matrix S) was tested using linear mixed-effects models (univariate

207   models for size parameters, shape indices and multivariate models for shape matrix S) :

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

209   where inner ear side (Si), growing degree day (GDD) and their interaction with dietary PUFA

210   content ($PUFA$) are fixed effects and individual (in) is introduced as a random effect

211    $(\Delta\alpha_{1,in})$ on the intercept to account for the fact that left and right otoliths' shapes are more

212   likely to be similar when originating from the same individual. The GDD values were

213   centered around the value at the beginning of the experiment (1 410 °C.day) so that the

214   intercept of the model represents otolith morphology at that point in time.

215   The multivariate versions of model (1) applied to shape matrix *S* were fitted while accounting

216   for variance heterogeneity between PCs (columns of *S*). Normality of the residuals and the

217   random effects as well as homoscedasticity of the residuals were assessed by visual inspection

218   of diagnostic plots. The models were fitted by maximizing the restricted log-likelihood. The

219   significance of the fixed effects was tested by likelihood ratio tests between nested models

220   while respecting marginality of the effects (type 2 tests; Fox and Weisberg, 2011) that are

221   supposed to follow a χ^2 distribution under the null hypothesis. Their p-values were adjusted

222   for Multiple Comparisons through the Bonferroni correction (McDonald, 2014).

223   To visualise shape differences between right and left otoliths, or DA amplitude, average left

224   and right shapes were rebuilt based on EFDs averaged for each side. DA amplitude was then

225   computed as the percentage of non-overlapping surface between the reconstructed right and

226   left otolith average shapes relative to the total area they covered after superposition.

227   2.4.2 Temperature and food composition effects on otolith morphogenesis

228 To test Temperature (T) and food composition ($PUFA$) effects on otolith morphogenesis, i.e.

229 the change with time in otolith shape O, four mixed-effects models (univariate or multivariate

230 depending on shape descriptors) were fitted:

231 $$O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})D + \alpha_2 D.T + \alpha_{3,PUFA}D + \alpha_{4,PUFA}D.T \tag{2}$$

232 $$O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})\,GDD + \alpha_{2,PUFA}GDD \tag{3}$$

233 $$O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})\,D + \alpha_2 D.GDD + \alpha_{3,PUFA}D + \alpha_{4,PUFA}D.GDD \tag{4}$$

234 $$O \sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})\,GDD + \alpha_{2,PUFA}GDD + \alpha_3 GDD.T + \alpha_{4,PUFA}GDD.T \tag{5}$$

235 where sampling day (D), dietary PUFA content ($PUFA$, 0.73% / 1.65%), temperature (T,

236 15°C / 20°C), growing degree day (GDD), and their interaction are fixed effects. PUFA was a

237 categorical variable in the 4 used models. In contrast, different options were applied to

238 account for the effect of temperature on otolith morphogenesis: either by considering

239 explicitly time D and temperature T (model 2) or by including a combined measure of thermal

240 time GDD (model 3) or by examining a combination of GDD and D (model 4) or GDD and T

241 (model 5) in case thermal time did not fully account for either real time effect or real

242 temperature effect. Random effects ($\Delta\alpha_{1,i}$) on D or GDD effect were used to account for

243 variability in otolith morphogenesis due to rearing tanks (i). These random effects affected

244 only the model slopes and not the intercepts because fish lived in the same tank until 93 dph,

245 i.e. the beginning of the full-factorial experiment at which $D = GDD = 0$.

246 We used the smallest Akaike's Information Criterion (AIC) to choose the most appropriate

247 model between the 4 options. Model fitting, diagnostics and significance tests of fixed effects

248 in the selected model were performed as described for DA analysis.

249 In a second step, individuals' total length TL was also included as a covariate in the selected

250 model that was re-fitted to estimate some potential confounding ontogenetic effects on otolith

251 shape, i.e. the fact that temperature or food composition could affect otolith shape indirectly

252 though their direct effect on fish body growth and the subsequent effect of fish body size on

253 otolith shape. Also, the potential sensibility of the results of these models to the threshold

254 temperature (Tth) used to compute GDDs was tested by varying the value of this parameter

255 from 0°C to 15°C by 1 degree increments and refitting the different models according to the

256 procedure described above.

257 Only the fixed part of the selected models is presented in the result section. Random effects,

258 which were included to account for variation due to tanks and thus to obtain robust estimates,

259 are presented in the Supplementary Material (Table S2). As for the DA analysis, to visualise

260 and to evaluate the amplitude of differences in otolith shape between pairs of groups

261 characterized by different rearing conditions, an average otolith shape by group was rebuilt

262 based on EFDs and the percentage of non-overlapping surface between reconstructed average

263 shapes of the two groups was calculated. Finally, the significance of shape difference between

264 all group pairs was tested using a mixed-effects model while correcting for multiple

265 comparisons using a Bonferroni procedure.

266 Statistical analyses were performed using the following packages in the statistical

267 environment R: 'nlme' (Pinheiro et al., 2016), 'lme4' (Bates et al., 2011), 'Vegan' (Oksanen

268 et al., 2013), 'car' (Fox et al., 2011) and 'MASS' (Venables and Ripley, 2002).

2. Results

3.1 Directional bilateral asymmetry (DA) in otolith shape

271 For all descriptors of otolith shape, the side value (i.e. the difference between the values

272 measured on the right otolith and on the left otolith), was always negative reflecting

measurements of the right otolith always lower than that of the left otolith (Table 1). According to the aspect of otolith morphology considered, the mixed-effects models did not show the same trend (Table 1). Regarding otolith size, represented by the four size parameters (Lo, Wo, Po and Ao), DA was significant since the beginning of the experiment (column Si, Table 1) but varied also according to experienced thermal opportunity (column $Si.GDD$, Table 1) and this differently depending on dietary PUFA content (column $Si.GDD.PUFA$, Table 1). Regarding otolith shape independent of size, represented by the other descriptors (Shape indices and EFDs), some DA was again detected since the beginning of the experiment (column Si, Table 1), except for rectangularity and the form-factor (lines Ore and Off, respectively, Table 1). However, in contrast with otolith size, DA in otolith shape did not vary with thermal opportunity (column $Si.GDD$, Table 1) nor with dietary PUFA content (column $Si.GDD.PUFA$, Table 1) The DA amplitude was on average equal to 1.17% throughout the experiment (Fig. 2). At 1410°C.day, the DA amplitude was 0.94% and increased with thermal opportunity but the values were higher at 15°C (2115°C.day: 1.31%; 3055°C.day: 1.36%) than at 20°C (2115°C.day: 1.04%; 3055°C.day: 1.08%). Given that DA in otolith shape was constant whatever thermal opportunity (GDD) and dietary PUFA content, only right otoliths were used in the following analyses.

3.2 Temperature and food composition effects on otolith morphogenesis

Among the four tested mixed-effects models, model (5) was always the best one according to the AIC whatever the response variable (Table 2). This model included GDD, $PUFA$, T and their interactions as fixed effects and applied similarly to fish somatic growth (i.e. fish total length as response variable), otolith growth (i.e. size parameters as response variables) and otolith morphogenesis (shape indices and matrix S as response variable). This suggests that thermal opportunity (GDD) was a better descriptor than the separate effects of time (D) and temperature (T) as represented in model 2. Thermal opportunity alone (model 3) was enough

298   to account for time effect but it was not to account for the full temperature effect, so that

299   interactions with time (D) were not necessary (model 4) but those with temperature (T) were

300   (model 5).

301   Despite better support for model 5 by AIC whatever the response variable, significance of the

302   different fixed effects varied across explained variables (Table 3). Fish growth (TL as

303   response variable) depended significantly on GDD but also on its interaction with T. Otolith

304   growth (size parameters as response variables, Table 3) depended significantly only on GDD

305   (response variables: Lo, Po, Wo) except for growth in otolith area (Ao) for which the

306   interaction with T was also significant. For otolith morphogenesis (shape indices and matrix S

307   as response variables, Table 3), results were more balanced with some indices (Oel, Ord and

308   Oar) indicating a significant effect of GDD only and others (Ore, Off and matrix S) also of

309   the interaction with T. It looked like the more complex was the index used to describe otolith

310   shape, the more likely to observe a significant interaction between GDD and T. In contrast,

311   the concentration of PUFA in diet had no significant effect on fish and otolith growth nor on

312   otolith morphogenesis (Table 3).

313   To test the effect of fish body size on the otolith shape, individual total length TL was

314   included as a covariate in model 5. There was no significant ontogenetic effect on the otolith

315   shape. . Moreover, the effect of the temperature threshold (Tth) was also tested in model 5.

316   While modifying the value of Tth from 0°C to 15°C by 1-degree increment, no changes

317   occurred in the significance of explanatory variables (Table S3).

318   The evolution of normalized otolith shape during the experiment showed that there were two

319   main changes which were related to the dorso-ventral axis and the area between the rostrum

320   and anti-rostrum (Fig. 3). The otolith shape was modified by a decrease of width/length ratio

321   and of the distance between the rostrum and the anti-rostrum as GDD and T increased. When

322   comparing average shapes observed for each condition and each sampling time to the first one

323   at 93 dph (Fig. 3), we observed that differences between temperatures at similar *GDD* were

324   only concerning the amplitude of the modification relative to the initial otolith shape (in other

325   terms, the speed of otolith morphogenesis) and not the direction of the modification (in other

326   terms the trajectory of otolith morphogenesis). At 20°C, otolith shape changed faster than at

327   15°C. This was evidenced by the percentage of non-overlapping surface between average

328   otolith shape at 93 dph and that at various points in "thermal time" later which was always

329   significantly higher at 20°C than at 15°C at the same "thermal time" (*GDD*; Table 4). In

330   contrast, the percentage of non-overlapping surface between average otolith shapes for

331   different dietary PUFA concentrations at the same *GDD* and *T* values was always lower than

332   2 % and was never significant (Table 4).

333   ## *3. Discussion*

334   ### *4.1 Directional bilateral asymmetry in seabass otolith*

335   In the present experiment, we found evidence of DA in otolith size reflecting measurements

336   of size parameters (length, width, area and perimeter) from the right otolith that are always

337   lower than that of the left otolith. The size of otoliths differed systematically (as opposed to

338   randomly) between right and left inner ears (e.g. side effect). The amplitude of DA varied

339   with GDD and n-3 PUFA concentration in diet. This indicates that the amplitude of the size

340   difference between left and right otoliths depends on thermal regime and diet quality during

341   the early-juvenile phase. Such directional bilateral asymmetry (DA) in shape (as opposed to

342   fluctuating asymmetry) is more important for flatfish than for roundfish because

343   metamorphosis causes unique asymmetric body shape and a lateralized behavior adapted to a

344   bottom-living lifestyle (Okada et al., 2001, Mille et al., 2015). However, significant DA has

345   been observed for roundfish species such as *Liza ramada* (Rebaya et al., 2017), *Diplodus*

346   *annulari*s (Trojette et al., 2015), *Diplodus puntazzo* (Bostanci et al., 2016), *Clupea harengus*

347   (Bird et al., 1986), *Scomberomorus niphonius* (Zhang et al., 2016) and *Merlangius merlangus*

348   (Mille et al., 2015). A recent study on *Boops boops* showed that, for this species, the

349   amplitude of DA varied geographically, at least partly due to environmental conditions (Mahé

350   et al., 2018). The variability of otolith DA in *Boops boops* at least could be related to

351   population geographical structuration and thus could be a phenotypically plastic response to

352   environmental drivers, such as temperature, current patterns and food availability, and/or

353   result from genetic differentiation between geographical locations. Moreover, the ontogenetic

354   trajectory of otolith shape is affected by environmental conditions during early life stages

355   (Vignon, 2018), thus DA and its amplitude could vary accordingly. Although we found a

356   significant DA of the otolith size, in size-normalized otolith shape, the DA of the otolith shape

357   was not significant, and it was not affected by the environmental temperature nor food

358   quality, suggesting that these two factors do not affect DA in otolith shape in juvenile seabass.

359   This study suggests that for seabass, considering a similar trajectory of otolith morphogenesis

360   between left and right sides, otolith growth rate is different which could reveal a functional

361   adaptation with lateralization of hearing functions.

362

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

364   Water temperature influences essential n-3 PUFA content in zooplankton and consequently its

365   availability to species at higher trophic levels such as fish (Gladyshev et al., 2011), which

366   may impair their growth. Skalli and Robin (2004) showed that, indeed, below a minimal

367   threshold (0.70%), dietary concentration of n-3 PUFA was positively correlated to mass

368   growth in European sea bass juveniles. In this study, our results showed that the effect of

369   dietary n-3 PUFA content on otolith morphogenesis was not linked to environmental

370   conditions, especially temperature. Using juvenile sea bass from the same experiment as the

371   present paper, Gourtay et al. (2018) found lower body growth and a significant decrease in n-

372   3 PUFA content in muscle tissues when fish oil was partly replaced by vegetable oil. The

373   dietary n-3 PUFA content also affected organ mass allometry with total mass for the heart and

374   the gastrointestinal system being smaller when temperature was high (20°C), whereas those of

375   liver and mesenteric fat were affected at lower temperatures (15°C) (Gourtay et al., 2018).

376   This suggests that environmental conditions, especially temperature, could also modify the n-

377   3 PUFA metabolism pathways in fish and thus, modulate several physiological functions, as

378   found in other species (Farkas et al., 1980; Olsen and Skjervold, 1995). Besides somatic

379   growth, Benitez-Santana et al. (2007) showed that the dietary n-3 PUFA could influence

380   swimming activities in gilthead seabream (*Sparus aurata*), which may be related to effects on

381   the sensory system. Dietary n-3 PUFA are clearly necessary for brain and retina development

382   during the first life stages of egg, larvae and juveniles (Bell and Dick, 1991; Furuita et al.,

383   1998). For example, it has been shown that an elevation of dietary DHA and EPA content

384   increases eye diameter of gilthead seabream larvae (Izquierdo et al., 2000). Similarly, dietary

385   n-3 PUFA could have a potential effect on the fish audio-vestibular system development and

386   especially on otolith biomineralisation, which relies on a minor organic fraction representing

387   0.2 to 10% of their total material (Degens et al., 1969). One hypothesis was that diet

388   composition may affect the synthesis of endolymph organic constituents by the saccular

389   epithelium, notably saccular endolymph proteins that are precursors of the otolith organic

390   matrix, and this probably conditions the biomineralisation process and the morphogenesis of

391   otoliths during the first life stages of fish. More specifically, n-3 PUFA, through their effect

392   on membrane viscosity, could affect the transport of the organic precursors in the endolymph

393   by the saccular epithelium and thus affect otolith biomineralisation. However, no evidence for

394   an effect of dietary n-3 PUFA content on the morphogenesis of otoliths in sea bass juveniles

395   was observed in the present study. Therefore, these results do not exclude a potential effect of

396   the dietary n-3 PUFA content on otolith morphogenesis below the minimal threshold.

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

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

Fish growth and temperature are always linked (Weatherley and Gill, 1987) because, over a mid-range of temperatures, somatic growth depends on metabolic enzyme activity, which is necessarily temperature dependent (Jobling, 1994). The GDD approach accounts for this relationship but assumes that growth is only a function of temperature and that this function is linear (Legg, 1998). However, there are other biotic or abiotic factors (e.g. population density, genetic make-up, food availability or composition, oxygen availability which may modulate the effect of temperature on fish growth. Under the assumption that temperature is the main contributor to growth, GDD can be a good descriptor of temperature-dependent processes that have been proven to be more relevant than calendar time and temperature value for growth in some fish species (Uphoff et al., 2013). Notably, Neuheimer and Taggart (2007) showed that variation in length-at-age among 17 stocks of North Atlantic cod (*Gadus morhua*) was significantly correlated with GDD and not with calendar time. Our study shows similar trend with seabass growth and otolith morphogenesis. However, the use of GDD required testing

423 potential effects of T values. Moreover, the effect of total length was tested in the models.

424 Similarly to Chezik et al. (2013), our study showed that the value of T (from 0°C to 15°C in 1

425 degree increments) did not significantly contribute to the model for fish length or otolith

426 shape. The value of T can bias the analysis only among populations, not within one population

427 (Chezik et al., 2013). In the same way, the models including GDD did not show any

428 interaction or influence with the fish total length covariate. Consequently, there was no direct

429 size-dependent effect on the otolith shape. Thus using GDD seems to be very appropriate to

430 test the effect of temperature on otolith morphogenesis.

431 ### *4.4 Effect of water temperature on the somatic growth and otolith shape*

432 The temperature effect, in this study, was explained by only GDD for several shape

433 descriptors (e.g. Lo, Po, Wo, Oel, Ord and Oar), and by GDD together with the GDD-T

434 relationship for other shape descriptors (e.g. Ao, Oci, Ore, Off and PCs). This difference

435 between the shape descriptors is explained by the level of details of the contour that is

436 integrated in the descriptor. Many of the commonly used descriptors (e.g. size parameters,

437 shape indices and EFDs) for otolith shape are correlated (Table S4). Hence, the number of

438 used descriptors could be decreased. In seabass, the otolith morphogenesis takes place in two

439 stages, firstly, a decrease of the width/length ratio and secondly, a modification on the surface

440 area between the rostrum and the anti-rostrum. In cod (*Gadus morhua*), the otolith shape

441 increases in crenulations and the number of lobes (Hüssy, 2008). Several shape descriptors

442 (length, perimeter, width, ellipticity, roundness and aspect ratio) are not able to describe this

443 type of development. Conversely, the weight or the volume of the otolith could be good

444 proxies to characterize otolith shape in three dimensions. Moreover, the reconstruction of

445 individual evolution of otolith shape could be used to better understand the biomineralisation

446 process and consequently the resulting otolith shape (Fablet et al., 2009).

447 Temperature affects fish growth through direct effects (e.g. metabolism) and indirect effects

448 (e.g. relationship with biotic factors such as availability of food) (Uphoff et al., 2013). In a

449 controlled experimental environment, we minimized indirect food effects. In the same way,

450 we tried to reduce the genetic background effect by using 4 females and 10 males to produce

451 the individuals used for this study. However, we cannot exclude individual genetic effects that

452 may have influenced the effect of temperature, since genetics directly affect the growth of

453 poikilotherms by influencing enzymatic activity and the stability of proteins configuration

454 and, therefore, the metabolic rates (Higley et al., 1986; Clarke and Johnston, 1999; Pauly,

455 2010). Fish exhibit higher growth rates when their metabolic activity rises with increasing

456 temperature (in mid-range temperatures, Clarke and Johnston, 1999). Our results on seabass

457 corroborated this observation and also showed the same trend for otolith growth. A previous

458 experimental study with zooplanktivorous age-0 walleye (*Sander vitreus*) had shown that food

459 consumption rates could also increase with increasing temperatures (Johnston and Mathias,

460 1994). Another recent study using carbon isotopic ratios with juvenile Australasian snapper

461 (*Chrysophrys auratus*) reared in the laboratory showed that the $\delta^{13}C$ in otoliths was not linked

462 to the dietary regime but rather temperature because of an overall increase of metabolic rates

463 in all fish tissues (Martino et al., 2019). Our study, which indicated that the effect of

464 temperature on otolith shape was the sum of GDD value and the interaction between GDD

465 and mean temperature, shows that the effect of temperature is to accelerate otolith

466 morphogenesis and to modify the evolution trajectory because at fixed GDD values, for any

467 two given temperatures, the otolith shapes are different.

468

Conclusion

470 Our results indicate that otolith shape is more sensitive to the environmental temperature than

471 to food quality (i.e. EPA and DHA contents) and, as such, can be an effective tool for

472 identifying fish stocks living in different thermal environments. Growing Degree Day (GDD)

473 is a very efficient tool to analyze the temperature effect on otolith growth. However, otolith

474 shape is also influenced by the relationship between GDD and water temperature. In fact,

475 otolith shape is significantly influenced by the cumulative sum of GDD, but the evolution

476 trajectory of the otolith morphogenesis is positively correlated with the temperature of the

477 environment. In fact,

478

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Table 1 : Statistical testing of directional bilateral asymmetry in otolith size and shape. p-values obtained in the mixed-effects models (1) for the effects of inner head side effect (Si) and their interactions with growing degree days (GDD) and food composition ($PUFA$) (Bonferroni corrected p-values for Multiple Comparisons: p-values < 0.0045: grey cases).

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

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 : *D+D:T+D:PUFA+ D:T:PUFA+i*	model 3 : *GDD+GDD:PUFA+i*	model 4 : *D+D:GDD+D:PUFA+D:G DD:PUFA+i*	model 5 : *GDD+GDD:PUFA+GDD :T+GDD:PUFA:T+i*
Fish growth	*TL*	94	332	293	0
Size parameters	*Lo*	28	168	144	0
	Po	39	161	140	0
	Ao	29	206	178	0
	Wo	27	190	158	0
Shape indices	*Ore*	94	332	304	0
	Oel	22	18	19	0
	Ord	18	1	4	0
	Oar	26	3	4	0
	Off	42	33	5	0
EFDs	*PCs*	3861	308	4087	0

758 *Table 3:* Results of mixed-effects models testing for the effects of food composition (*PUFA,*
759 0.73%/1.65%), experimental temperature (*T*, 15°C/20°C), growing degree day (*GDD*), and
760 their interaction for each response variable (Adjusted P-values for Multiple Comparisons: p-
761 values < 0.0045: **).
762

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

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

	non-overlap (%)	1410 °C.day	2155 °C.day				3055 °C.day			
		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	▬

Figure 1: Main steps of the experimental design for seabass with 5 samplings at 93, 129, 141, 176 and 204 dph. Each group (G) was composed of 3 replicates. To test the temperature effect, 15 °C (blue) and 20 °C (red) were used and for concentrations of PUFA, 1.85 (dotted) and 0.85 (striated) were used.
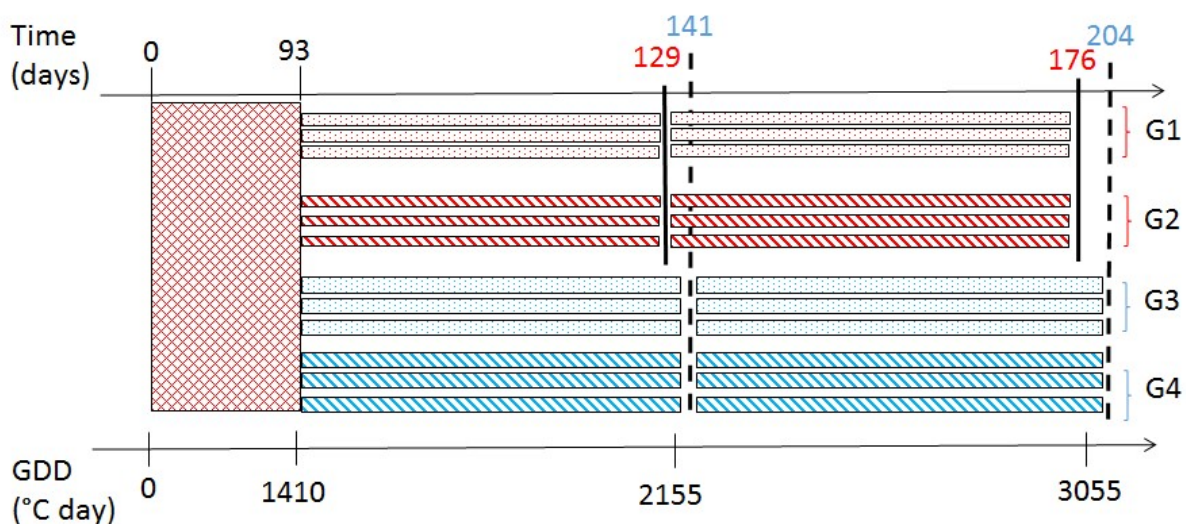
Figure 2: Difference between *left (black dotted line) and right (grey line) average otolith shape.*
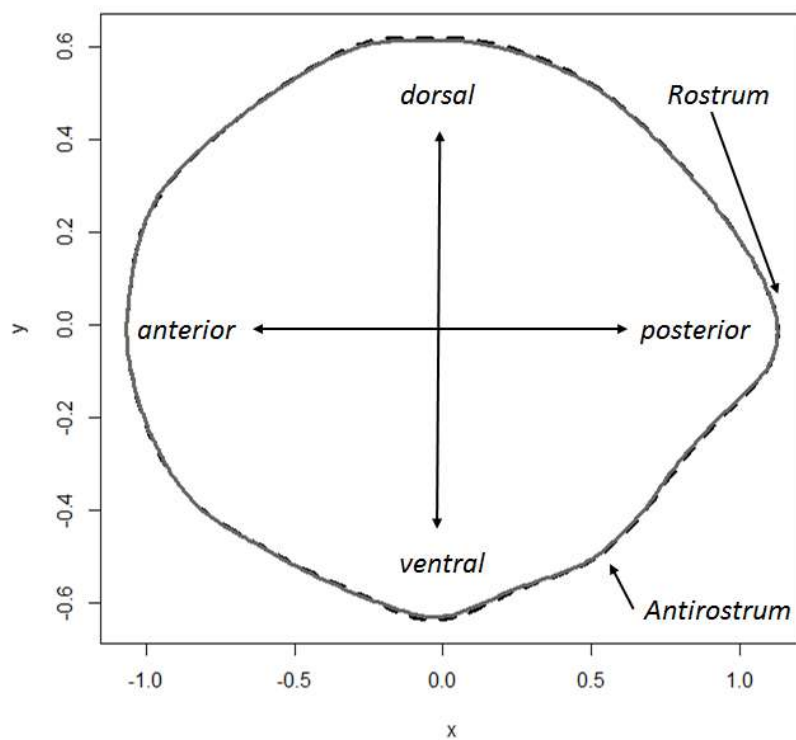

Figure 3: Evolution of otolith normalized shape during the experiment: D-93 (1410°C day; black line), D-129 (2155°C day; 20°C; red dashed line), D-146 (2155°C day; 15°C; blue dashed line), D-176 (3055°C day; 20°C; red line) and D-204 (3055°C day; 15°C; blue line).

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