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.

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).

Otoliths are calcified structures located in the inner ear of teleost fish that are characterized by accretional growth and are metabolically inert (i.e. once deposited, otolith material is unlikely to be resorbed or altered) (Casselman, 1987). Otolith shape is influenced by both abiotic (e.g. temperature) and biotic (e.g. food availability) environmental parameters and depends on individuals’ genotype (Vignon and Morat, 2010). Since otolith growth is related to fish growth (Mahé et al., 2017) and otolith shape co-varies with fish diet in the wild (Mille et al. 2016), global climate change may affect otolith morphogenesis and growth through its effects on both temperature and n-3 PUFA availability. As stocks are characterized by different environmental conditions and genetic makeup, associated variation in otolith growth, which generates variation in otolith shape, may allow stock discrimination along species distributional range (Cadrin et al., 2014). In fact, the otolith shape is commonly used as a tool
to identify species and to discriminate between fish stocks. Since Campana and Casselman’s seminal work (1993), many fishery scientists have used this approach for stock discrimination studies in order to understand fish population dynamics and achieve reliable exploited fish stock assessments for fishery management (Reiss et al., 2009). Therefore, it is crucial to identify environmental variations that could influence otolith morphogenesis and growth. In addition, the shape of otoliths could differ systematically (as opposed to randomly) between right and left inner ears (e.g. side effect). A recent study on Boops boops showed that, for this species, the amplitude of directional bilateral asymmetry (DA) varied geographically, at least partly due to environmental conditions (Mahé et al., 2018).

2. The present study investigates under controlled conditions the crossed effects of temperature (15°C and 20°C) and dietary n-3 PUFA content (0.73% EPA+DHA versus 1.65% EPA+DHA) on otolith morphogenesis and growth in European seabass. We investigated how otolith morphological variability within individuals, i.e. directional bilateral asymmetry, and between individuals is affected by environmental drivers, more precisely water temperature and dietary n-3 PUFA content, during the early life of seabass. This species is commercially important for both fisheries and aquaculture in Europe, especially in the Mediterranean Sea. The world aquaculture production of European seabass was 191,003 tons in 2016 (FAO FishStat data). The effects of temperature or dietary n-3 PUFA concentration on seabass somatic growth have been well-studied for aquaculture purposes (Person-Le Ruyet et al., 2004a, 2004b; Skalli and Robin, 2004), but their effects on otolith morphogenesis and growth are currently unknown. Materials and methods

2.1. Fish and rearing conditions

Seabass adults were captured and then reared in captivity for three years, after which a batch of 4 females and 10 males was used to obtain the eggs for this study. The experiment was carried out in 2017 using 93-day-old juveniles that were offspring of adult European seabass captured in winter 2013 in the Gulf of Morbihan (France). At 2 days post hatching (dph), seabass larvae were divided among three conical tanks (230 L, 10μm filtered seawater, UV,
salinity 35‰, initial density 10 000 larvae tank⁻¹). Water temperature in the tanks was progressively increased from 14°C to 20°C from 2 to 8 dph. From the mouth opening stage to 39 dph, larvae were fed with Artemia. At 40 dph, larvae were co-fed with Artemia and a microparticulate diet (Marinstart, Le Gouessant, Lamballe, France) for four days, and then fed exclusively with the microparticulate diet until 74 dph. Thereafter, juveniles were fed with larger pellets for ornamental fish (EPA + DHA = 1.5%, Le Gouessant, Lamballe, France) until the beginning of the experiment at 93 dph.

2.2. Experimental design

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

To follow-up the otolith growth and shape variation through time, fish were sampled 3 times over the course of the experiment. At 93 dph, 30 individuals were sampled and then at the other two samplings, 10 fish per tank (i.e. 30 fish per each experimental condition and each at each time) were also sampled. To analyze the temperature effect on growth and shape of the otolith, we used the “heat units approach” that has been developed since about 1730 (e.g., Réaumur, 1735), initially to quantify the thermal opportunity for the growth and the
development of plants by aggregating temperatures relevant to these processes. Designated by a number of expressions, including “thermal time”, “heat units”, “thermal units”, or “growth units”, thermal opportunity for growth is usually measured in “growing degree days” (GDD; Wang, 1960; Bonhomme 2000; Trudgill et al., 2005; Neuheimer and Taggart; 2007) that are calculated as follows:

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

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

2.3. **Otolith shape analysis and measures**

After measuring the total length (TL± 0.1 cm) of fish, their sagittal otoliths (left and right) were extracted from the cranial cavity and cleaned. In order to minimize distortion errors in the normalization process during image analysis, each otolith was placed on a microscope slide with the sulcus facing downward and the rostrum pointing left. The outline of each otolith was digitized using an image analysis system consisting of a high-resolution camera
(Hamamatsu Orca 3G) connected to an automated microscope using reflected light (Zeiss Axio Imager Z2). Each digitized image was analyzed using the image-analysis software TNPC (Digital processing for calcified structures, version 7). To compare the shapes of the left and right otoliths, mirror images of the right otoliths were used. Otolith shape was assessed by analyzing three types of descriptors: the otoliths’ size parameters (Length: \(L_o\); Width: \(W_o\); Perimeter: \(P_o\); Area: \(A_o\)), shape indices (Table S1) and Elliptic Fourier Descriptors (EFDs; e.g. Lestrel, 2008). Size parameters are measurements directly related to the size of the otolith, unlike shape indices, which are dimensionless measurements and therefore independent of the size of the otolith. The shape indices measure otolith morphology similarity to ideal geometric shapes calculated using size parameters (Table S1): Roundness (\(Ord\)) and Form-factor (\(Off\)) quantify similarity to a circle, Ellipticity (\(Oel\)) to an ellipse, and Apect-Ratio (\(Oar\)) and Rectangularity (\(Ore\)) to a rectangle. EFDs were obtained by using TNPC 7 software to perform Fourier analysis on binarized image. For each otolith, the first 99 elliptical Fourier harmonics (H) were extracted and normalized with respect to the first harmonic so as to be invariant to otolith size, rotation and starting point of contour description (Kuhl and Giardina, 1982). To determine the number of harmonics required to reconstruct the otolith outline, the cumulated Fourier Power (\(F\)) was calculated for each otolith \(k\) as a measure of the precision of contour reconstruction obtained with \(n_k\) harmonics (i.e., the proportion of variance in contour coordinates accounted for by the \(n_k\) harmonics):

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

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

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

2.4 Statistical analyses

2.4.1 Directional bilateral asymmetry in otolith shape

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

\[
O \sim \alpha_0 + \Delta \alpha_{0,in} + \alpha_1 Si + \alpha_2 GDD + \alpha_3 Si \cdot GDD + \alpha_4 PUFA Si \cdot GDD
\]  

(1)

where inner ear side (Si), growing degree day (GDD) and their interaction with dietary PUFA content (PUFA) are fixed effects and individual (in) is introduced as a random effect \((\Delta \alpha_{1,in})\) on the intercept to account for the fact that left and right otoliths’ shapes are more likely to be similar when originating from the same individual. The GDD values were centered around the value at the beginning of the experiment (1410 °C.day) so that the intercept of the model represents otolith morphology at that point in time.
The multivariate versions of model (1) applied to shape matrix $S$ were fitted while accounting for variance heterogeneity between PCs (columns of $S$). Normality of the residuals and the random effects as well as homoscedasticity of the residuals were assessed by visual inspection of diagnostic plots. The models were fitted by maximizing the restricted log-likelihood. The significance of the fixed effects was tested by likelihood ratio tests between nested models while respecting marginality of the effects (type 2 tests; Fox and Weisberg, 2011) that are supposed to follow a $\chi^2$ distribution under the null hypothesis. Their $p$-values were adjusted for Multiple Comparisons through the Bonferroni correction (McDonald, 2014).

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

2.4.2 Temperature and food composition effects on otolith morphogenesis

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

\begin{align}
O &\sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})D + \alpha_2D.T + \alpha_{3,PUFA}D + \alpha_{4,PUFA}D.T \\
O &\sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})GDD + \alpha_{2,PUFA}GDD \\
O &\sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})D + \alpha_2D.GDD + \alpha_{3,PUFA}D + \alpha_{4,PUFA}D.GDD \\
O &\sim \alpha_0 + (\alpha_1 + \Delta\alpha_{1,i})GDD + \alpha_{2,PUFA}GDD + \alpha_{3}GDD.T + \alpha_{4,PUFA}GDD.T
\end{align}

where sampling day ($D$), dietary PUFA content ($PUFA$, 0.73% / 1.65%), temperature ($T$, 15°C / 20°C), growing degree day ($GDD$), and their interaction are fixed effects. PUFA was a categorical variable in the 4 used models. In contrast, different options were applied to
account for the effect of temperature on otolith morphogenesis: either by considering explicitly time $D$ and temperature $T$ (model 2) or by including a combined measure of thermal time $GDD$ (model 3) or by examining a combination of $GDD$ and $D$ (model 4) or GDD and $T$ (model 5) in case thermal time did not fully account for either real time effect or real temperature effect. Random effects ($\Delta \alpha_{1,i}$) on $D$ or $GDD$ effect were used to account for variability in otolith morphogenesis due to rearing tanks ($i$). These random effects affected only the model slopes and not the intercepts because fish lived in the same tank until 93 dph, i.e. the beginning of the full-factorial experiment at which $D = GDD = 0$.

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

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

Only the fixed part of the selected models is presented in the result section. Random effects, which were included to account for variation due to tanks and thus to obtain robust estimates, are presented in the Supplementary Material (Table S2). As for the DA analysis, to visualise and to evaluate the amplitude of differences in otolith shape between pairs of groups characterized by different rearing conditions, an average otolith shape by group was rebuilt.
based on EFDs and the percentage of non-overlapping surface between reconstructed average shapes of the two groups was calculated. Finally, the significance of shape difference between all group pairs was tested using a mixed-effects model while correcting for multiple comparisons using a Bonferroni procedure.

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

3. Results

3.1 Directional bilateral asymmetry (DA) in otolith shape

For all descriptors of otolith shape, the side value (i.e. the difference between the values 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 \(\text{(Lo, Wo, Po and Ao)}\), DA was significant since the beginning of the experiment (column \(\text{Si}_{.\text{GDD}}\), Table 1) but varied also according to experienced thermal opportunity (column \(\text{Si}_{.\text{GDD} . \text{PUFA}}\), Table 1) and this differently depending on dietary PUFA content (column \(\text{Si}_{.\text{GDD} . \text{PUFA}}\), 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 \(\text{Si}\), Table 1), except for rectangularity and the form-factor (lines \(\text{Ore}\) and \(\text{Off}\), respectively, Table 1). However, in contrast with otolith size, DA in otolith shape did not vary with thermal opportunity (column \(\text{Si}_{.\text{GDD}}\), Table 1) nor with dietary PUFA content (column \(\text{Si}_{.\text{GDD} . \text{PUFA}}\), 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 to account for time effect but it was not to account for the full temperature effect, so that interactions with time (D) were not necessary (model 4) but those with temperature (T) were (model 5).

Despite better support for model 5 by AIC whatever the response variable, significance of the different fixed effects varied across explained variables (Table 3). Fish growth (TL as response variable) depended significantly on GDD but also on its interaction with T. Otolith growth (size parameters as response variables, Table 3) depended significantly only on GDD (response variables: Lo, Po, Wo) except for growth in otolith area (Ao) for which the interaction with T was also significant. For otolith morphogenesis (shape indices and matrix S as response variables, Table 3), results were more balanced with some indices (Oel, Ord and Oar) indicating a significant effect of GDD only and others (Ore, Off and matrix S) also of the interaction with T. It looked like the more complex was the index used to describe otolith shape, the more likely to observe a significant interaction between GDD and T. In contrast,
the concentration of PUFA in diet had no significant effect on fish and otolith growth nor on
otolith morphogenesis (Table 3).
To test the effect of fish body size on the otolith shape, individual total length TL was
included as a covariate in model 5. There was no significant ontogenetic effect on the otolith
shape. Moreover, the effect of the temperature threshold (Tth) was also tested in model 5.
While modifying the value of Tth from 0°C to 15°C by 1-degree increment, no changes
occurred in the significance of explanatory variables (Table S3).
The evolution of normalized otolith shape during the experiment showed that there were two
main changes which were related to the dorso-ventral axis and the area between the rostrum
and anti-rostrum (Fig. 3). The otolith shape was modified by a decrease of width/length ratio
and of the distance between the rostrum and the anti-rostrum as GDD and T increased. When
comparing average shapes observed for each condition and each sampling time to the first one
at 93 dph (Fig. 3), we observed that differences between temperatures at similar GDD were
only concerning the amplitude of the modification relative to the initial otolith shape (in other
terms, the speed of otolith morphogenesis) and not the direction of the modification (in other
terms the trajectory of otolith morphogenesis). At 20°C, otolith shape changed faster than at
15°C. This was evidenced by the percentage of non-overlapping surface between average
otolith shape at 93 dph and that at various points in “thermal time” later which was always
significantly higher at 20°C than at 15°C at the same “thermal time” (GDD; Table 4). In
contrast, the percentage of non-overlapping surface between average otolith shapes for
different dietary PUFA concentrations at the same GDD and T values was always lower than
2 % and was never significant (Table 4).
4. Discussion

4.1 Directional bilateral asymmetry in seabass otolith

In the present experiment, we found evidence of DA in otolith size reflecting measurements of size parameters (length, width, area and perimeter) from the right otolith that are always lower than that of the left otolith. The size of otoliths differed systematically (as opposed to randomly) between right and left inner ears (e.g. side effect). The amplitude of DA varied with GDD and n-3 PUFA concentration in diet. This indicates that the amplitude of the size difference between left and right otoliths depends on thermal regime and diet quality during the early-juvenile phase. Such directional bilateral asymmetry (DA) in shape (as opposed to fluctuating asymmetry) is more important for flatfish than for roundfish because metamorphosis causes unique asymmetric body shape and a lateralized behavior adapted to a bottom-living lifestyle (Okada et al., 2001, Mille et al., 2015). However, significant DA has been observed for roundfish species such as Liza ramada (Rebaya et al., 2017), Diplodus annularis (Trojette et al., 2015), Diplodus puntazzo (Bostanci et al., 2016), Clupea harengus (Bird et al., 1986), Scomberomorus niphonius (Zhang et al., 2016) and Merlangius merlangus (Mille et al., 2015). A recent study on Boops boops showed that, for this species, the amplitude of DA varied geographically, at least partly due to environmental conditions (Mahé et al., 2018). The variability of otolith DA in Boops boops at least could be related to population geographical structuration and thus could be a phenotypically plastic response to environmental drivers, such as temperature, current patterns and food availability, and/or result from genetic differentiation between geographical locations. Moreover, the ontogenetic trajectory of otolith shape is affected by environmental conditions during early life stages (Vignon, 2018), thus DA and its amplitude could vary accordingly. Although we found a significant DA of the otolith size, in size-normalized otolith shape, the DA of the otolith shape was not significant, and it was not affected by the environmental temperature nor food
quality, suggesting that these two factors do not affect DA in otolith shape in juvenile seabass. This study suggests that for seabass, considering a similar trajectory of otolith morphogenesis between left and right sides, otolith growth rate is different which could reveal a functional adaptation with lateralization of hearing functions.

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

Water temperature influences essential n-3 PUFA content in zooplankton and consequently its availability to species at higher trophic levels such as fish (Gladyshev et al., 2011), which may impair their growth. Skalli and Robin (2004) showed that, indeed, below a minimal threshold (0.70%), dietary concentration of n-3 PUFA was positively correlated to mass growth in European sea bass juveniles. In this study, our results showed that the effect of dietary n-3 PUFA content on otolith morphogenesis was not linked to environmental conditions, especially temperature. Using juvenile sea bass from the same experiment as the present paper, Gourtay et al. (2018) found lower body growth and a significant decrease in n-3 PUFA content in muscle tissues when fish oil was partly replaced by vegetable oil. The dietary n-3 PUFA content also affected organ mass allometry with total mass for the heart and the gastrointestinal system being smaller when temperature was high (20°C), whereas those of liver and mesenteric fat were affected at lower temperatures (15°C) (Gourtay et al., 2018). This suggests that environmental conditions, especially temperature, could also modify the n-3 PUFA metabolism pathways in fish and thus, modulate several physiological functions, as found in other species (Farkas et al., 1980; Olsen and Skjervold, 1995). Besides somatic growth, Benitez-Santana et al. (2007) showed that the dietary n-3 PUFA could influence swimming activities in gilthead seabream (Sparus aurata), which may be related to effects on the sensory system. Dietary n-3 PUFA are clearly necessary for brain and retina development.
during the first life stages of egg, larvae and juveniles (Bell and Dick, 1991; Furuita et al., 1998). For example, it has been shown that an elevation of dietary DHA and EPA content increases eye diameter of gilthead seabream larvae (Izquierdo et al., 2000). Similarly, dietary n-3 PUFA could have a potential effect on the fish audio-vestibular system development and especially on otolith biomineralisation, which relies on a minor organic fraction representing 0.2 to 10% of their total material (Degens et al., 1969). One hypothesis was that diet composition may affect the synthesis of endolymph organic constituents by the saccular epithelium, notably saccular endolymph proteins that are precursors of the otolith organic matrix, and this probably conditions the biomineralisation process and the morphogenesis of otoliths during the first life stages of fish. More specifically, n-3 PUFA, through their effect on membrane viscosity, could affect the transport of the organic precursors in the endolymph by the saccular epithelium and thus affect otolith biomineralisation. However, no evidence for an effect of dietary n-3 PUFA content on the morphogenesis of otoliths in sea bass juveniles was observed in the present study. Therefore, these results do not exclude a potential effect of 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 potential effects of $T$ values. Moreover, the effect of total length was tested in the models. Similarly to Chezik et al. (2013), our study showed that the value of $T$ (from 0°C to 15°C in 1 degree increments) did not significantly contribute to the model for fish length or otolith shape. The value of $T$ can bias the analysis only among populations, not within one population (Chezik et al., 2013). In the same way, the models including GDD did not show any interaction or influence with the fish total length covariate. Consequently, there was no direct size-dependent effect on the otolith shape. Thus using GDD seems to be very appropriate to test the effect of temperature on otolith morphogenesis.
4.4 Effect of water temperature on the somatic growth and otolith shape

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

Temperature affects fish growth through direct effects (e.g. metabolism) and indirect effects (e.g. relationship with biotic factors such as availability of food) (Uphoff et al., 2013). In a controlled experimental environment, we minimized indirect food effects. In the same way, we tried to reduce the genetic background effect by using 4 females and 10 males to produce the individuals used for this study. However, we cannot exclude individual genetic effects that may have influenced the effect of temperature, since genetics directly affect the growth of poikilotherms by influencing enzymatic activity and the stability of proteins configuration and, therefore, the metabolic rates (Higley et al., 1986; Clarke and Johnston, 1999; Pauly, 2010). Fish exhibit higher growth rates when their metabolic activity rises with increasing temperature (in mid-range temperatures, Clarke and Johnston, 1999). Our results on seabass
Corroborated this observation and also showed the same trend for otolith growth. A previous experimental study with zooplanktivorous age-0 walleye (Sander vitreus) had shown that food consumption rates could also increase with increasing temperatures (Johnston and Mathias, 1994). Another recent study using carbon isotopic ratios with juvenile Australasian snapper (Chrysophrys auratus) reared in the laboratory showed that the $\delta^{13}$C in otoliths was not linked to the dietary regime but rather temperature because of an overall increase of metabolic rates in all fish tissues (Martino et al., 2019). Our study, which indicated that the effect of temperature on otolith shape was the sum of GDD value and the interaction between GDD and mean temperature, shows that the effect of temperature is to accelerate otolith morphogenesis and to modify the evolution trajectory because at fixed GDD values, for any two given temperatures, the otolith shapes are different.

Conclusion

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

5. Acknowledgements

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6. References


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).

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<th>Si.GDD.PUFA</th>
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Table 2: Δ AIC for 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).

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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:**).

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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).

<table>
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<tr>
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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).
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).
Amplitude difference (\%) 12%

Temperature
- 15°C
- 20°C

Growing Degree Days
- 1410°C.day
- 2155°C.day
- 3055°C.day

+ 645 °C.day
+ 900 °C.day

Food: Polyunsaturated Fatty Acid (% PUFA)
- 1.65
- 0.73

Dicentrarchus labrax

ΩMEGA 3