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**Freshwater fish otoliths record signals from both water and physiological processes: new insights from Sr/Ca and Ba/Ca ratios**

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## ABSTRACT

Using strontium and barium in otoliths to determine natal origins and understand patterns of fish movements is based on the fundamental assumption that otoliths record water chemistry signals without any major alterations. Although prior studies highlighted that fish physiology can modify the water signal in otolith, studies for freshwater fish are scarce. We exposed different group of Atlantic salmon parr *Salmo salar* to different scenarios of ambient-level variations in Sr/Ca and Ba/Ca ratios and then combined otolith chemical profiles with environmental data (water chemistry, temperature), Fulton's index and otolith growth rates to assess what factors explain/influence the elemental ratios of Sr and Ba in otoliths. Generalized additive mixed-models (GAMMs) using water-based otolith composition, temperature, Fulton's index and 'individual' as explanatory variables, allow to demonstrate that water chemistry alone cannot fully explain measured ratios in otoliths, except in scenarios involving significant changes in water chemistry. Others factors (physiological effects) should be accounted for reproducing short and minimal seasonal variations in water composition and considering that inter-individual variability contributed quite significantly in most scenarios.

**Keywords:** Otolith chemistry; Otolith growth rate; Atlantic salmon; Freshwaters; Field and experimental studies.

## INTRODUCTION

Examining otolith elemental records for reconstructing fish population structure, connectivity and movement patterns, has emerged as a reliable method complementary to tagging and genetic studies (Walther et al., 2010). Otoliths are composed of calcium (Ca) carbonate structures that grow throughout the life of the fish, and incorporate chemical elements derived from both extrinsic (environmental) and intrinsic (physiological dynamics) sources

(Campana et al. 1985; Campana, 1999; Woodcock et al., 2012; Sturrock et al., 2012, 2014, 2015). As otolith expand, new Ca carbonate layers are formed. Lattice vacancies present in any crystal structure are then being filled with trace elements via extrinsic and intrinsic inputs. This unique feature constitutes the basis for reconstructing fish life-histories. However, to achieve a reliable reconstruction across habitats, we need to associate changes in otolith elemental records with either signals induced by environmental conditions (temperature, ambient water composition) and/or signatures resulting from biological processes (growth rate, diet, metabolism) (Campana, 1999; Campana and Thorrold, 2001; Walther, 2019). In addition, this approach must be reliable throughout the entire fish life and applicable for all locations visited by the fish. Unfortunately, disentangling between environmental and biological influences on the element-otolith association remains challenging, particularly when examining fish otoliths from freshwater systems. Prior studies characterized the factors controlling elemental incorporation into otoliths mostly by focusing on fish from estuarine and marine environments through experimental approaches (Sturrock et al., 2012; Hüsey et al., 2020). Taken together, the main finding is the complexity of the system as several mechanisms are involved. Otolith records a combination of chemical (water salinity), physical (water temperature) and biological (growth rate) factors, as well as specific binding pathways related to each species (Eldson and Gillanders, 2003; Barnes and Gillanders, 2013; Sturrock et al., 2014, 2015; Stanley et al., 2015; Izzo et al., 2018). Surprisingly, most experiments were conducted with fish exposed either to constant water chemical and physical parameters or to unrealistic transitions (Sturrock et al., 2012), making the data collected difficult to interpret (Sturrock et al., 2015; Izzo et al., 2018). This may be particularly problematic for studies conducted in freshwater systems where we can expect the water chemical composition to display a stronger heterogeneity than marine waters. Previous works found contrasted outcomes on how temperature affects the elemental uptake of marine fish otoliths. A first group of studies reported an increase of the Sr/Ca ratio with

temperature (Martin et al., 2004; Collingsworth et al., 2010; Miller, 2011; Webb et al., 2012; Reis-Santos et al., 2013; Walsh and Gillanders, 2018), a second group observed an opposite trend with the Sr/Ca ratio decreasing as temperature rises (Radtke, 1989; Radtke et al., 1990; Townsend et al., 1995; Martin and Wuenschel, 2006; DiMaria et al., 2010; Marohn et al., 2011) while no significant effect of temperature on the Sr/Ca and Ba/Ca ratios could be found by a third group of studies (Gallahar and Kingsford, 1996; Gaudie, 1996; Chesney et al., 1998; Walther et al., 2010; Miller and Hurst, 2020). The diet composition may affect the Sr/Ca and Ba/Ca ratios in otoliths yet there is still no consensus regarding the direction (Gallahar and Kingsford, 1996; Hoff and Fuiman, 1995; Collingsworth et al., 2010; Webb et al., 2012; Doubleday et al., 2013; Miller and Hurst, 2020). Additionally, a certain inertia between variations in the chemical water composition and subsequent changes in otolith microchemistry were documented suggesting a potential metabolic influence (Elsdon and Gillanders, 2005; Macdonald and Crook, 2010; Yokouchi et al., 2011; Miller, 2011). Finally, ontogenetic variations are likely to affect otolith chemistry regardless of environmental conditions (De Pontual et al., 2003; Kalish, 1989).

The Sr/Ca and Ba/Ca ratios have been used to determine the displacement or natal origins of anadromous and freshwater fish (Wells et al., 2003; Brown and Severin, 2009; Doubleday et al., 2013; Martin et al., 2013a; Teichert et al., 2018). However, despite possible confounding effects, the influence of temperature, diet and growth rate on the incorporation of Sr and Ba were rarely investigated in freshwater environments (Collingsworth et al., 2010; Miller, 2011; Doubleday et al., 2013). When examined for freshwater systems, relationships between water chemical composition and elemental otolith records were only investigated for short time-periods (Teichert et al., 2018) or over extended periods of life (Martin et al., 2013a). The fundamental assumption made when using Sr/Ca and Ba/Ca ratios is that otoliths record water chemistry signals without any major alterations. Although prior studies focusing on

marine systems highlighted that fish physiology can modify the water signal in otolith, similar evidences for freshwater fish are scarce. Thus, it is crucial to properly assess how the water chemistry affects the otolith capacity to record environmental changes in freshwater systems.

The two aims of our study were i) to assess whether Sr/Ca and Ba/Ca ratios in otoliths can reliably reflect different life-history scenarios in salmon parr and ii) to verify if other variables (i.e. temperature, fish condition factor and individual variability) were able to explain/influence deviations between observed and water-based predicted Sr/Ca and Ba/Ca ratios. Several scenarios simulating an array of chemical changes, including steep decrease or increase, transient increase and slight seasonal oscillations in water Sr/Ca and Ba/Ca ratios, were tested using generalized additive mixed-models (GAMMs, Lin and Zhang, 1999) and correlation tests (Table 1).

## **MATERIALS AND METHODS**

### **Otolith sampling**

A first group of twenty-nine otoliths were selected from fish that spent their early life in two hatcheries (Medous and Sassis) during fry stage (Table 1). Fish were collected in 2006, 2009, and 2010 on sites along the Gave de Pau River (Table 1, Figure S1) using a standardized electrofishing method. Twenty-two parr were classified as age-0<sup>+</sup> and seven as age-1<sup>+</sup> years. Fry held at a relatively constant water temperature (8-10°C) in the Medous hatchery were released April 15<sup>th</sup> of 2005, 2006, 2009 and 2010 in the Gave de Pau River — 2 weeks after yolk-sac resorption. Fry at the Sassis hatchery were kept at constant temperature (12°C) for a longer period of time and artificially fed for about 3 months with marine-derived pellets (Biomar, Nersac, France), before their release in the Gave de Pau River on June 15<sup>th</sup> of 2009. In addition, six to ten fries from the two hatcheries were also collected in 2009 and their otoliths examined for chemical profiles.

A second group of otoliths was also obtained from a one hundred thirty-nine-day experiment conducted in 2011 at the Lapitxuri field-station (Martin, 2013; ECP, INRAE, 2018) located on a tributary of the Nivelle River (France) (1°29'W, 43°17'N; Figure S1). Briefly, Atlantic salmon embryos (forty-eight-day-old) conceived by wild genitors from the Bidassoa River were incubated for sixty-two days in a flow-through incubator before the fry were transferred to a Lapitxuri experimental channel for seventy-seven additional days under natural conditions (i.e. photoperiod, water chemistry, temperature, natural prey found in the channel). Following hatching in the flow through incubator (day fifty-five), fry experienced a flood event on day ninety. Larvae were thus exposed to a 40% increase in the Ba/Ca ratio for ~ three weeks (days ninety to one hundred and eleven). The Ba/Ca ratio then constantly decreased until day one hundred thirty-two to finally remain relatively stable during the last two months of the experiment —without returning exactly to pre-flood values. The Sr/Ca ratio stayed constant during the overall experiment. To identify the transition period between hatching and release dates in the Lapitxuri experimental channel, two Alizarin Red S (C<sub>14</sub>H<sub>7</sub>O<sub>7</sub>SNa, Sigma-Aldrich Corporation, Saint Quentin Fallavier, France) fluoromarking immersions were carried out. The first was performed shortly post-hatching on day sixty and the second on day one hundred and ten, i.e. five to ten days after larvae exhausted their vitelline reserve but before fry were transferred to the experimental channel. One hundred and sixty fish survived the experiment. However, large variation in the fork lengths was then observed (mean L<sub>F</sub>: 53.5 ± 6.1 mm; range: 39-71 mm; Figure S2). To account for the size effect, twenty-four otoliths of one hundred eighty-seven-day-old salmon juveniles were selected for otolith chemistry in three different size classes: eight with L<sub>F</sub> between [35-45] mm, nine with L<sub>F</sub> between [50-55] mm and seven with L<sub>F</sub> between [60-75] mm (Table 1, Figure S2).

## Water sampling and analysis

Water samples were collected in 2010 every two weeks over a two month-period at the Medous hatchery (n=5, mid-February to mid-April) and over a four month-period at the Sassis hatchery (n=8, mid-February to mid-June). Water sampling was conducted every week at the Lapitxuri field-station (n=18) from January 17, 2011 to June 1, 2011. Water chemistry was also monitored in the Gave de Pau River every week in 2007 (n=31), every month in 2010 (March to August, n=7) and on more scattered dates in 2009 and 2011 years (n=7). The 2007 dataset covered the entire hydrological cycle and were part of a water pollution-monitoring program of trace metals that received extra funding. Temperature (°C) and pH were also recorded at the time of each water withdrawal in the Gave de Pau (2007, 2009, 2010, 2011). Water temperature was measured daily every two hours at the Lapitxuri experimental station.

Water samples were filtered through a PVDF Whatmann 0.45 µm filters with syringes into 125 ml acid-washed LDPE (low-density polyethylene) bottles and acidified (2%) using ultrapure nitric acid HNO<sub>3</sub> (JT Baker, Ultrex II). All plastic materials used for sample collection were acid-washed prior to use. Samples were immediately transferred to a cooler and refrigerated upon return to the laboratory.

Filtered water samples were analysed for calcium (Ca), strontium (Sr) and barium (Ba) concentrations using ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometry - ACTIVA, *Jobin Yvon*) using external calibration (Martin et al., 2013a). Certified reference materials (SLRS-5, NRCC, Canada – SRM1640A, NIST, USA) were analysed every ten samples. Precision and accuracy were determined by analysing reference materials ( $n = 48$ ) and were all well within 3% of the targeted value except the precision for Ba/Ca that was slightly higher (<10%).

## Otolith analyses



Otoliths were processed using the protocol described in Martin et al. (2013a). Briefly, one *sagitta* per fish was embedded in epoxy resin (Araldite 2020, Escil) with the primordial surface downwards. Resin blocks were ground gradually with ultrapure water and sandpaper (1200– 4000 grit) until the primordium was reached and polished with diamond-tipped polishing powder. Finally, otoliths were carefully rinsed with ultrapure-water, and then air-dried before being stored in individually labelled plastic vials.

Detection of Alizarin Red S marks on otoliths collected at the Lapitxuri experiment was carried out using an Eclipse 90i compound microscope (Nikon, Champigny sur Marne, France), equipped with a Nikon G-2A filter cube (excitation filter: 510–560 nm / dichromatic mirror: 565 nm / barrier filter: 590). Digital images of each structure were recorded using the Nikon digital camera DXM1200C. Otoliths were carefully examined under transmitted light at 400× and 1000× magnifications to check for hatch and yolk absorption marks. Distances from the otolith core to the two Alizarin Red S marks were measured. The second otolith of some Lapitxuri parr was prepared on a transverse plan for daily growth rate analysis. For this purpose, otoliths were embedded in epoxy resin Araldite 2020 (Escil, France) and ground on the transversal plane with abrasive papers (2 400 grit) to expose the daily growth increments until satisfactory readability and polished with diamond solutions (0.25 µm; Escil; Chassieu, France). Otolith photographs were taken and assembled into a large image using Photostitch software (Canon; [www.canonutilities-photostitch.en.lo4d.com](http://www.canonutilities-photostitch.en.lo4d.com)). Using ImageJ (NIH, USA), fish growth increments were counted along the profile from the core to the outer edge, and the distance between each count was incremented to estimate daily growth rates. Counts were performed twice by two independent readers.

Cleaned otoliths were analysed for Sr, Ba, and Ca concentrations using an Elan DRC 2 (Perkin Elmer, USA) inductively coupled plasma quadrupole mass spectrometer (ICP-MS) coupled to a high repetition rate Infra-Red femto-second laser ablation system (Alfamet-

Novalase, France; Martin et al., 2013a). Strontium and Ba concentrations were then expressed as molar ratios to calcium in  $\text{mmol.mol}^{-1}$  for Sr/Ca and  $\mu\text{mol.mol}^{-1}$  for Ba/Ca on the basis of the stoichiometry of Ca carbonate ( $9.73 \text{ mmol Ca.g}^{-1}$  otolith) (Brown and Severin, 2009).

## Data treatment and statistical analysis

Predicted Sr/Ca and Ba/Ca otolith profiles (water-based otolith composition) were calculated by multiplying partition coefficients ( $D_{\text{element}}$ ) with the water chemical composition. Partition coefficients for Sr/Ca ( $D_{\text{Sr}}$ ) and Ba/Ca ( $D_{\text{Ba}}$ ) ratios have been shown to be influenced by water concentration, temperature, diet and growth rates (DiMaria et al., 2010; Miller et al., 2020). We calculated  $D_{\text{Sr}}$  and  $D_{\text{Ba}}$  from otolith and water data from thirteen rivers covering a relatively broad spectrum of Sr/Ca and Ba/Ca ratios (Table 2; Martin et al., 2013a,b). Both  $D_{\text{Sr}}$  and  $D_{\text{Ba}}$  were obtained using chemical ratios of the outer edge of otoliths (i.e., the last 100  $\mu\text{m}$  that represents about one to two months of life) and the average water ratios of the corresponding summer months. We assumed the effects of temperature and growth across the rivers are limited and that predicted otolith signals must be in agreement with the chemical otolith ratio recorded during that summer period. Because no significant trend was found between  $D_{\text{Sr}}$  and the Sr/Ca water composition (Figure S3), the average value of the thirteen rivers was used,  $D_{\text{Sr}} = 0.26 \pm 0.05$  (Table 2). Conversely, as the  $D_{\text{Ba}}$  decreases exponentially with the water composition (Figure S3), three average values were defined (Table 2): one for the lowest water ratios ( $D_{\text{Ba}} = 0.026 \pm 0.008$  that was used for the Medous and Sassis hatcheries), one for intermediate ratios ( $D_{\text{Ba}} = 0.024 \pm 0.006$  that was used for the Gave de Pau River) and finally one for the highest water ratios ( $D_{\text{Ba}} = 0.017 \pm 0.005$  that was used for the Lapitxuri experiment). The uncertainty on the partition coefficients ( $\Delta D_{\text{element}}$ ) was determined from standard deviation (SD) from the chemical composition of waters and otoliths according to the following equation:

$$\Delta D_{Me} = [(\sigma_{Me}^{otolith} / \bar{X}_{Me}^{otolith}) + [(\sigma_{Me}^{water} / \bar{X}_{Me}^{water})] \times D_{Me} \quad (1)$$

where Me corresponds to the Sr/Ca or Ba/Ca ratios. Predicted average, minima and maxima for Sr/Ca and Ba/Ca ratios were calculated and compared with the measured signals (Figures 1, 2, 3, 4 and 5). We made the assumption that deviation from the prediction was significant when the measured signal was below the minima or above the maxima. Because the water database from the Gave de Pau River was collected over several years, a certain degree of heterogeneity can be observed, yet a general annual trend can also be detected. We thus decided to process all data as an integrative and smoothed curve representative of a median annual hydrological cycle. As such, a polynomial regression fitting was applied to the data to predict seasonal changes in Sr/Ca and Ba/Ca water patterns into the Gave de Pau River (Figure S4).

For each individual, raw elemental values (Sr/Ca or Ba/Ca, mmol.mol<sup>-1</sup>), water-based prediction of otolith composition and temperature were reported to the number of days since egg deposition (Lapitxuri field-experiment) or to a relative temporal scale corresponding to the age of parr in months. Well-defined temporal anchor points, i.e. alizarin marks for the Lapitxuri mesocosm experiment and breakpoints related to habitat changes of known calendar date (hatcheries to Gave de Pau River), were used to delineate segments where a constant growth rate was applied for a given individual. For age-1<sup>+</sup> parr, the annual growth mark was used to better constrain the otolith chemical signals as a function of time. The two known temporal markers (two fluorescent marks: AM1 and AM2) at 60 and 110 days post egg deposition, respectively, were used on otoliths of juveniles reared at the Lapitxuri field-station to transform distances (µm) on otolith chemical profiles to days by assuming constant growth rate on each interval. In the case of hatchery-reared individuals stocked in the Gave de Pau River, we used otolith <sup>87</sup>Sr/<sup>86</sup>Sr profiles (Martin et al., 2013a) to delimit the Gave de Pau habitat from that of

Sassis hatchery. Strontium isotopic signatures for this site (0.7092-0.7095) were indeed significantly different from the one for the Gave de Pau River (0.710-0.7103) (Martin, 2013). For the Medous hatchery, comparison of parr profiles to those of fry collected in this hatchery allowed us to identify changes in habitat as both Sr/Ca and Ba/Ca ratios exhibit rapid and significant changes following stocking. Assuming constant growth rate through time may not be optimal and could lead to time deviation for some otolith chemical profiles compared to others at least between the anchor points (Sturrock et al., 2015); however, we think it is a reasonable assumption for our study.

The Fulton's index (for the entire fish life) and the otolith growth rate (OGR; for specific periods of time) were determined. The Fulton's index (K-factor) was determined to estimate the body condition using the following equation (Fulton, 1904):

$$K = 100 * W/L^3 \quad (2)$$

Where W is the weight in g and L the length in cm.

Average OGR were calculated for age-0<sup>+</sup> parr by dividing the distance between anchor points by the number of associated days both for the Gave de Pau development period (from the date of stocking until collection in the fall) and the Lapitxuri experiment (AM2-edge). OGR of two specific periods during the experimentation in the Lapitxuri channel, i.e. the flood event and the normal flow conditions during the last month, were determined by averaging daily growth rates.

Individual raw elemental values (Sr/Ca and Ba/Ca, mmol.mol<sup>-1</sup> and μmol.mol<sup>-1</sup>, respectively) were modeled using generalized additive mixed-models (GAMM, Lin and Zhang, 1999) implemented using the *mgcv* package (version 1.8-24) in R 3.2.2 (Wood, 2017). Various models were fitted for each life-history scenario, including varying fixed and random effects. While the null model (m0) only contains an intercept, the saturated model (m1= full model) contains the following explanatory variables: i) Water-based prediction of otolith composition

(mean-value,  $\text{mmol.mol}^{-1}$  for Sr/Ca and  $\mu\text{mol.mol}^{-1}$  for Ba/Ca), ii) water temperature ( $^{\circ}\text{C}$ ), and iii) individual Fulton's index. Other models were submodels of the saturated one and consisted in all possible combinations of one, two or three variables (see Table S1 for further details on models' formula). This approach allows to capture potential non-linear trends between the elemental composition of otoliths and the explanatory variables. In these mixed-models, water-based otolith composition (linear trend), water temperature (non-linear trend), and Fulton's index (linear trend) are treated as a fixed effect. In contrast, 'individuals' were treated as random effects (i.e. factor-smoother interaction between individuals and water-based prediction of otolith composition within *mgcv*), allowing for the baseline value of elemental composition (i.e. smoothness parameter) to vary for each fish. Given the presence of a random effects, the restricted maximum likelihood (REML) estimator was preferred for fitting models (Reiss and Ogden, 2009; Wood, 2011). Akaike information criterion (AIC) is a robust approach to compare the different model structures, accommodating the effects of penalization and smoothing parameter uncertainty (Wood et al. 2016). Here, Akaike weight was used for model selection. Akaike weights represent the relative likelihood of a model. The Akaike weight is a value between 0 and 1, with the sum of Akaike weights of all models in the candidate set being 1, and can be considered as analogous to the probability that a given model is the best approximating model. In all the datasets, full saturated models appeared among the most likely ones (Table S2) and were therefore subsequently used. Full models are described as follow:

“Otolith\_Composition = Water-based otolith composition +  $f(\text{Water-based otolith composition})$  +  $f(\text{Temperature})$  + Fulton's +  $\zeta_{\text{individuals}}$  +  $\epsilon_i$ ”, where  $f(x)$  is a smooth function of the covariate  $x$ ,  $\zeta_{\text{individuals}}$  is the random effect for fish [smoothness parameter of  $f(\text{Water-based otolith composition})$  allowed to vary for each fish] and  $\epsilon_i$  is a Gaussian error term. Summary, including fixed terms and approximate significance of smooth terms, of the full saturated model for each scenario is reported in supplementary material (Table S3).

The fit of the full model was appraised by comparing the overall deviance explained by the full model with deviance explained by a null model (intercept only) as:

$$\frac{deviance_{(null\ model)} - deviance_{(full\ model)}}{deviance_{(null\ model)}} \quad (3)$$

Likewise, partial deviance explained by each variable in the full model was estimated by:

$$\frac{deviance_{(model\ without\ the\ explanatory\ variable)} - deviance_{(full\ model)}}{deviance_{(null\ model)}} \quad (4)$$

These calculations are used to determine the relative importance of each predictor (in terms of variance). Summed partial deviance explained for explanatory variables did not always add up to the total deviance explained due to concurvity (i.e. the fact that one smooth term can be approximated by some combination of the other smooth terms in the model. In other words, due to the overlap in the variance explained by different variables within the same model).

To test the role of individual OGR for explaining the large inter-individual variability of Sr/Ca and Ba/Ca ratios during specific periods (equilibrium stage, unexpected peaks, flood event), Pearson or Spearman correlation tests were conducted depending on the data normality according to the Shapiro-Wilk normality test. Statistical analyses were carried out using R software version 3.2.2.

**RESULTS**

**Elemental otolith composition: Measured vs water-based predictions**

Predicted Sr/Ca and Ba/Ca ratios are shown as average (bold), minima (dotted) and maxima (dotted) lines over the entire fish life for qualitative comparison with all the measured profiles of each individual parr according to their capture site (Gave de Pau River, Lapitxuri channel) and their hatchery of origin (Medous, Sassis). Overall, Sr/Ca and Ba/Ca ratios in otoliths usually effectively represent changes in water chemical composition (Fig. 1, 2, 3, 4 and 5). However,

the measured ratios in otoliths differed significantly from the predicted ratios for specific periods of time (Sr/Ca: every winter; Ba/Ca: following the stocking or in response to the flood event) and depending on the individual.

#### *Sr/Ca ratio*

A significant decrease in  $\text{Sr/Ca}_{\text{otolith}}$  occurred when parr experienced a steep decrease in the water Sr/Ca ratio, i.e. when they were moved from the Sassis hatchery to the Gave de Pau River (Fig. 1). Variability was observed among individuals for the time required for the  $\text{Sr/Ca}_{\text{otolith}}$  to reach a new equilibrium, ranging from one month to two and a half months, resulting in significant differences between the measured and predicted ratios when individuals were transferred from their original habitat to another. Beside that episode, the predicted Sr/Ca ratios generally matched the measured ratios.

For the individuals only subjected to slight seasonal fluctuations (Medous moved to the Gave de Pau River), the measured  $\text{Sr/Ca}_{\text{otolith}}$  generally partially matched the predicted trend from water chemistry (Fig. 2a,b). Surprisingly, fry reared at the Medous hatchery exhibited maximal values for  $\text{Sr/Ca}_{\text{otolith}}$  during their early life in the Gave de Pau (age-0<sup>+</sup>, Fig.2a) but also during their second winter (age-1<sup>+</sup>, Fig. 2b,c), while water chemical composition displayed minimal ratio for the same time period (Fig. 2b; Figure S4). The measured  $\text{Sr/Ca}_{\text{otolith}}$  was slightly higher than Sr/Ca ratio predicted from the water chemistry during the stay at the Medous hatchery, probably because of the contribution of Sr-enriched diet. Furthermore, although seasonal variation of  $\text{Sr/Ca}_{\text{water}}$  was only ~7%, seasonal changes in measured Sr/Ca ratio in otoliths were ~23-37%.

#### *Ba/Ca ratio*

Following significant increases –three and six-fold increase for larvae from the Medous and Sassis hatcheries, respectively– in water Ba/Ca ratio, all otolith profiles for age-0<sup>+</sup> parr display maxima values coinciding with the fish's release in the Gave de Pau River (Fig. 3a, b). These maxima were relatively variable depending on the individual, and usually occurred within one to two weeks after the stocking date (Fig. 3a, b) and were poorly predicted by the water-based otolith composition. Unlike the Sr/Ca ratio, only one maximum in Ba/Ca<sub>otolith</sub> was observed for age-1<sup>+</sup> parr again following the fish's release in the Gave de Pau River (Fig. 4). Measured Ba/Ca<sub>otolith</sub> signals reasonably matched the predictions throughout the remaining profile but fine-scale variations did not perfectly match the fluctuations of the chemical composition of the water (Fig. 4).

No otolith response to small (40%) and short period of time (3 weeks) increases of the water Ba/Ca ratio at the Lapitxuri River, was detected immediately after changes in the water chemistry (Fig. 5). An increase in Ba/Ca<sub>otolith</sub> was generally detectable after day 110 suggesting a ~20 days delayed response for the otolith to properly record a change in water signal. Additionally, as in the Gave de Pau River, the Ba/Ca<sub>otolith</sub> profiles displayed a wide variation regardless of the size classes, some otolith transects being characterized by high Ba/Ca<sub>otolith</sub> maxima (up to ~7  $\mu\text{mol}\cdot\text{mol}^{-1}$ ) well over predicted values while others displayed no major peaks but a rather gradual increase more in accordance with water-based predictions (Fig. 5). Following the flood response, Ba/Ca<sub>otolith</sub> gradually decreased to a relatively stable value (Fig. 5) but the dispersion among individuals remained still high (Table 3, CV = 15-17%). Finally, predicted and measured mean values were in relatively good agreement during normal flow at the end of the experiment for all size classes (Table 3).

### Contribution of covariables based on GAMMs



Very good fits (>90% of deviance explained) were found for only two scenarios: (1) age-0<sup>+</sup> with steep Sr/Ca decrease and (2) six-fold Ba/Ca increase. For the three other scenarios involving age-0<sup>+</sup> individuals, GAMMs only fit 66%, 72% and 80% of the deviance, for slight Sr/Ca increase, three-fold and transient Ba/Ca increase, respectively (Table 4). The covariates used could only explain less than half of the deviance in scenarios where fishes of age-1<sup>+</sup> were mainly subject to slight seasonal variations in Sr/Ca and Ba/Ca chemical ratios (Table 4), with only 42% and 28% of deviance explained, respectively. GAMMs applied to our data clearly highlight that interindividual variability (independently from individual condition index) significantly contributed to the Sr/Ca and Ba/Ca variations in otoliths of salmons for all scenarios — with contribution being at least as important as water-based prediction (Table 4). Given that the mean value was used for the water-based otolith composition, the contribution of ‘interindividual variability’ is therefore probably overestimated. Temperature seems a poor predictor in explaining Sr/Ca and Ba/Ca variations in otoliths, yet this explanation cannot be totally discarded because temperatures partially correlate with water composition in our datasets.

#### **OGR vs interindividual variability**

##### *Sr/Ca ratio*

For age-0<sup>+</sup> parr from the Gave de Pau, the delay to reach Sr/Ca<sub>otolith</sub> equilibrium ratio during the summer period presented a large range of individual responses and was not significantly correlated to the average OGR during the stay in the Gave de Pau regardless of the hatchery of origin ( $r=-0.14$ ,  $p\text{-value}=0.52$ , Figure S5). Sr/Ca<sub>otolith</sub> ratios above the Sr/Ca ratio predicted during the winter period (Fig. 2a,b) were not significantly correlated to the OGR ( $r=-0.08$ ,  $p\text{-value}=0.65$ ). At equilibrium, Sr/Ca<sub>otolith</sub> was relatively constant between individuals (CV: 11%)

and unrelated to the OGR ( $r=-0.20$ ,  $p\text{-value}= 0.33$ ) and the somatic growth rate ( $r=-0.31$ ,  $p\text{-value}= 0.13$ ).

#### *Ba/Ca ratio*

Among age-0<sup>+</sup> parr, the lag to reach Ba/Ca<sub>otolith</sub> equilibrium value correlates significantly with the average OGR during their stay in the Gave de Pau River whether individuals were previously reared in the Medous or Sassis hatcheries ( $r=-0.61$ ,  $p\text{-value}= 0.0012$ , Figure S6). However, this delay was not significantly linked to outlier's value ( $r=-0.04$ ,  $p\text{-value}= 0.85$ ). Ultimately, Ba/Ca<sub>otolith</sub> during the maxima or at equilibrium were not significantly correlated with the average OGR ( $r=-0.059$  with  $p\text{-value} = 0.78$  and  $r=-0.14$  with  $p\text{-value} = 0.51$ , respectively) and somatic growth rate ( $r=-0.36$  with  $p\text{-value} = 0.11$  and  $r=-0.14$  with  $p\text{-value} = 0.38$ , respectively).

Regarding the controlled experiment, the ratio of Ba/Ca<sub>otolith</sub> during the flood episode correlated significantly with the OGR average for individuals that stayed in the Lapitxuri channel ( $r = 0.74$ ,  $p\text{-value} = 0.001$ ), but not significantly with the OGR recorded during the flood ( $r = 0.48$ ,  $p\text{-value} = 0.10$ ). No significant relationship was found between the Ba/Ca<sub>otolith</sub> equilibrium value and the OGRs for the Lapitxuri experiment ( $r = 0.32$ ,  $p\text{-value} = 0.24$ ) or the normal flow period ( $r = 0.17$ ,  $p\text{-value} = 0.58$ ). On average, individuals of the [50-55] and [60-75] size classes were more prone to accumulate higher Ba/Ca in their otolith than the lowest size class [35-45] (Table 4) during the flood episode (multiple comparison Dunn test:  $p\text{-value} = 0.0052$  for [50-55] vs [35-45] and  $p\text{-value} = 0.0109$  for [60-75] vs [35-45]). Predicted Ba/Ca<sub>otolith</sub> during the flood episode better reflected individuals from the smallest size class [35-45] (Fig. 5, Table 3), while individuals growing faster ([50-55] and [60-75] size classes) over-accumulated the Ba quite significantly, well above predicted values (Fig. 5). Overall, a wide range of Ba/Ca<sub>otolith</sub> profiles was observed regardless of the size classes. On average, individuals of the [50-55] and

[60-75] size classes were more prone to accumulate higher Ba/Ca in their otolith than the lowest size class [35-45] (Table 3) during the flood episode (multiple comparison Dunn test: *p-value* = 0.0052 for [50-55] vs [35-45] and *p-value* = 0.0109 for [60-75] vs [35-45]).

## DISCUSSION

Our study consisted in examining fish otolith response among freshwater systems exhibiting contrasted or slightly variable water chemistry while allowing factors like temperature, diet and physiology to vary. We acknowledge that our approach might be challenging for isolating specific effects driven by temperature, food or growth rate. Modeling otolith variations (via GAMM) with three explanatory variables treated as fixed effect (water-based prediction of otolith composition, temperature, Fulton's index) and 'individual' treated as random effect, allowed us to demonstrate that water chemistry alone cannot fully explain measured ratios in otoliths, except in scenarios involving significant changes in water chemistry (Table 4). Furthermore, individual variability partly explains the otolith variations in most scenarios, even if its contribution may be overestimated by our approach (the use of only mean-value for the water-based otolith composition). Additionally, the covariates included in the models did not fully or poorly explain the observations in some scenario (30-80% of deviance explained), especially when fish were exposed to slight seasonal variations in Sr/Ca and Ba/Ca water ratios or limited increase in Ba/Ca water ratios (Table 4). Taken together our new results are partially in agreement with previous studies indicating that Sr and Ba otolith composition of Atlantic salmon roughly reflects changes in freshwater systems (Wells et al., 2003; Martin et al., 2013a; Carlson et al., 2017). Indeed, although we collected evidence that water chemistry clearly drove significant shift in otolith composition when there is a significant change in water chemistry, we also found that additional factors (intrinsic, i.e. growth rate, metabolism, and/or extrinsic, i.e. temperature) may lead to discrepancies between otolith elemental records and water-based

otolith prediction when habitat chemistry varies slightly. When facing identical/synchronous environmental changes, the large inter-individual variation in chemical profiles may support the combined influence of intrinsic factors (e.g., physiology) as previously suggested for marine fish (Brown and Severin, 2009; Sturrock et al., 2014, 2015).

#### **Parameters influencing seasonal variations in $\text{Sr}/\text{Ca}_{\text{otolith}}$**

Seasonal variations in otolith  $\text{Sr}/\text{Ca}$  ratios were already examined for various fish species. They are generally related to changing habitats especially for species known to perform seasonal migrations between chemically distinct habitats such as fresh and marine waters (Brown and Severin, 2009; Hüsey et al., 2020). In some circumstances, however, habitat change cannot be invoked to explain variation in the otolith elemental composition and other factors must be considered (Sturrock et al., 2015; Loewen et al., 2016; Hüsey et al., 2020). Our study demonstrated that seasonal fluctuations in  $\text{Sr}/\text{Ca}$  were captured by otoliths for parr from the Gave de Pau River. It is highly unlikely that these fluctuations depend exclusively on changes of the water chemistry since fish were only exposed to very slight variations ( $\sim 7\%$ ) whereas otolith  $\text{Sr}/\text{Ca}$  ratios significantly exceeded the predicted ratio during low growing periods ( $37 \pm 11\%$  and  $23 \pm 12\%$ , respectively for the first and second winter). Seasonal variations in  $\text{Sr}/\text{Ca}_{\text{otolith}}$  associated with the opaque (growth season) or translucent (winter period) zones of otoliths were already reported and related to mixed influence of temperature and growth rate (Sturrock et al., 2015; Hüsey et al., 2020). In this study, otolith  $\text{Sr}/\text{Ca}$  ratios for salmon parr varied seasonally in the opposite direction to water temperature and water chemistry.

Our results collected from the Gave de Pau River showed that otolith  $\text{Sr}/\text{Ca}_{\text{otolith}}$  values of Atlantic salmon parr decreased with higher water temperature values, in contradiction with one of the few studies based on another freshwater species (*Perca flavescens*, Collingsworth et al. 2010). Answering whether water temperature influences Sr incorporation in otoliths of

salmon parr remains a challenge and additional work is still required. Nonetheless, we found a variation of about 0.1-0.15 mmol.mol<sup>-1</sup> between winters and summer periods, which is about the same order of magnitude as for the variation produced by a 10°C temperature change on eel Sr/Ca<sub>otolith</sub> (0.2 mmol.mol<sup>-1</sup>: Marohn et al., 2011). Temperature effects were also reported to be consistently lower than environmental gradient experienced by the fish when changing habitat (Hüssy et al., 2020). The 0.1-0.15 mmol.mol<sup>-1</sup> seasonal fluctuations we observed was low in comparison to the overall range of Sr/Ca<sub>otolith</sub> encountered among habitats in the Adour basin (0.29 to 0.95 mmol.mol<sup>-1</sup>, Table 2).

The results of our study support, in addition to the water chemistry, a mixed physiological/temperature influence on Sr/Ca<sub>otolith</sub> in freshwater systems as recently underpinned for some marine species (Sturrock et al., 2014, 2015; Grammer et al, 2017; Hüssy et al., 2020). Using a one year-long experiment involving monthly measurements of both environmental and physiological parameters, Sturrock et al. (2014, 2015) showed that sex and reproductive status of the European plaice (marine fish species) triggered specific variations in the Sr/Ca composition of the otolith unrelated to the surrounding water. Further, Izzo et al. (2018) have conducted a meta-analysis (85% from marine species) combining water composition, otolith chemistry and some environmental parameters. They clearly emphasized the influence of temperature and salinity, as well as the role of intrinsic factors (growth, diet) that can act either independently or interactively. Mixed models combining field biogeochemical tracers with fish growth and otolith chemistry also revealed that Sr/Ca ratio is affected by physiological effects (growth, assimilation rates) variable through time (Grammer et al., 2017) in the marine environment. Additionally, Barnes and Gillanders (2013) were able to demonstrate that genetics had a significant interactive effect with environmental factors (temperature and salinity) on both Sr/Ca and Ba/Ca ratios within otoliths.

While there is still no consensus, recent findings suggested that growth negatively influences Sr/Ca composition in blood plasma and otolith for marine fish (Secor and Rooker, 2000; Marohn et al., 2011; Daverat et al., 2012; Stanley et al., 2015; Sturrock et al., 2014, 2015; Miller et al., 2020). However, controlled laboratory experiments found no relationship between growth rate and Sr/Ca<sub>otolith</sub> (Hoff and Fuiman, 1995; DiMaria et al., 2010; Marohn et al., 2011). Additionally, how food ration and composition would affect otolith Sr remains evasive (Walther et al., 2010; Hoff and Fuiman, 1995; Gallahar and Kingsford, 1996). In salmon parr, higher Sr otolith concentrations typically occur in translucent zones under transmitted light (autumn-winter low growth period) while lower levels appear in opaque zones (growth season –spring to mid-summer, Bacon et al., 2005; Strothotte et al., 2005), consistent with a negative effect of growth. Growth is connected to temperature variations and involves multiple physiological pathways, which result in the production and the transport of organic substances in the blood and thus to the endolymph, subsequently influencing the transport and incorporation of chemical elements into the otolith aragonite (Gronkjaer, 2016). In addition, growth can act on the incorporation of elements into the otolith through kinetic dilution effects related to crystal accretion rates, high calcification rates resulting in lower Sr/Ca incorporation into otolith (Campana, 1999; Hamer et al., 2007; Hüsey et al., 2020). A fraction of Sr likely binds to protein complexes in the blood and endolymph fluids (Funamoto and Mugiya, 1998; Thomas et al., 2017). Although data are scarce, as high as 2/3 of the Sr was reported to be protein-bound in the plasma of the goldfish *Carassius auratus* (Funamoto and Mugiya, 1998). Furthermore, a positive relationship was demonstrated in the blood plasma between Sr concentration and protein content for a marine fish (Sturrock et al., 2014), suggesting Sr availability to the otolith can be strongly controlled by physiological variations. Strontium availability in the blood plasma (Sturrock et al., 2014) and the endolymph fluid could thus be lower during the growing season as higher concentrations of organic substances are expected

in these fluids (Hüssy and Mosegaard, 2004; Gronkjaer, 2016). We favor a model where otolith Sr/Ca fluctuations recorded in salmon parr would result from interactive effects of both temperature and physiology, whether through the control of available Sr by organic molecules in the blood and the endolymph or kinetic effects on element incorporation during the growing season.

Finally, time variability observed for individuals to reach a new equilibrium (from high Sr/Ca ratio values to lower values) supports a significant influence of the fish's metabolism. However, Sr/Ca otolith was not significantly related to both somatic growth rate and average OGR in our study. Before their sequestration in otoliths, dissolved ions go through several biochemical processes across various membranes that ultimately alter their original bioavailability (Kalish 1989, 1991, Campana 1999, Payan et al. 2004, Melancon et al. 2009). A multitude of physiological and biochemical mechanisms are probably involved in the continuously forming otolith and can explain the wide range in individual responses for a given freshwater system.

#### **Parameters influencing the variations in Ba/Ca<sub>otolith</sub>**

When exposed to different water chemistry, we found the Ba incorporation in otolith of parr was generally delayed by ~2-3 weeks. Our findings are similar to previous experimental studies showing that there was a significant time lag ( $\geq 20$  d) between variations in water chemical composition and subsequent changes in otolith microchemistry (Elsdon and Gillanders, 2005; Lowe et al., 2009; Macdonald and Crook, 2010; Yokouchi et al., 2011). Elemental incorporation in otoliths is a multi-barrier process (Campana, 1999). Barium requires more time than other elements before being precipitated onto the otolith surface (Elsdon and Gillanders, 2003) suggesting a strong biological control. Miller (2011) estimated that changes in otolith

composition of juveniles Chinook salmon were detected within two to three days of a change in water chemistry but otolith compositions did not stabilize until fifteen days.

Laboratory controlled experiments were inconclusive regarding the influence of water temperature on the  $Ba/Ca_{\text{otolith}}$ . Some studies reported small positive effects (Miller, 2009; Reis-Santos et al., 2013; Stanley et al., 2015; Walsh and Gillanders, 2018), negative effects (DiMaria et al., 2010; Sturrock et al., 2015) or no significant effects (Martin and Thorrold, 2005; Martin and Wuenschel, 2006; Miller et al., 2020). The effects of food ration and growth rate were tested and demonstrated an inverse response for  $Ba/Ca_{\text{otolith}}$  (Walther et al., 2010; Miller, 2011; Sturrock et al., 2015), but no significant relationship was found between growth rate and  $Ba/Ca_{\text{otolith}}$  for larval fish (Bath et al., 2000; Martin and Thorrold, 2005). Interactions between temperature, diet and growth rate in the field make it difficult to separate their effects. Indeed, increasing temperatures typically result in increased ration ingestion and growth rates.

A wide scattering was found in  $Ba/Ca_{\text{otolith}}$  following an increase of  $Ba/Ca_{\text{water}}$  for individuals that experienced similar environmental conditions, thus ruling out an environmental cause (water chemistry composition and temperature), but suggesting variable physiological effects with a level-response specific to each individual. A similar variable response in otolith  $Ba/Ca$  ratio was previously observed in wild salmon juveniles during the transition from the yolk sac to the surrounding environment before the final equilibrium value was reached several weeks later (Martin et al., 2013a). Peak values at this transition appeared unrelated to ambient  $Ba/Ca$  ratios because they did not significantly change among fishes that experienced low / high  $Ba/Ca_{\text{water}}$  ratios (Martin et al., 2013a). Other field-based studies that examined fish movements from the sea (low  $Ba/Ca_{\text{water}}$ ) into lower salinity environments (high  $Ba/Ca_{\text{water}}$ ) reported significant maxima in  $Ba/Ca_{\text{otolith}}$  (Hale and Swearer, 2008). A laboratory experiment also reported euryhaline Australian bass *Macquaria novemaculeata* exhibiting a steep unpredictable increase in  $Ba/Ca_{\text{otolith}}$  immediately after their transfer into a low salinity system (Macdonald



and Crook, 2010). Substantial and consistent two- to three-fold variations in metabolic rate that were linked with variations in behaviour and critical life-history traits were demonstrated among individuals of a same species (Norin et al., 2015; Norin and Gamperl, 2017). In case where  $Ba/Ca_{\text{otolith}}$  is mainly controlled by metabolic rate, a broad response among individuals exposed to unique environmental condition would be expected. As early life stages are subject to intense physiological processes (de Pontual et al., 2003) with possible ontogenetic asynchrony between individuals, it can explain the overall large variation in Ba/Ca ratio among age-0<sup>+</sup>.

We propose that a sudden increase in water Ba concentration could trigger physiological responses, inducing a progressive regulation of Ba incorporation into otolith following an overdone response relatively variable depending on the individual physiology (Kalish, 1989; Sadovy and Severin, 1994). Our study, as well as previous observations, isolate the following pattern: short-time high and variable Ba uptake / progressive exponential decrease in the Ba uptake / stabilization of the ratio which can take weeks to months.

Our controlled-experiment conducted at the Lapitxuri station showed that fast-growing individuals accumulated more Ba than slower growing individuals during the flood event and that  $Ba/Ca_{\text{otolith}}$  correlated significantly with the overall OGR during the stay in the channel ( $r = 0.75$ ,  $p$ -value = 0.001) but poorly with the specific OGR during the flood ( $r = 0.48$ ,  $p$ -value = 0.10). Surprisingly, no correlation was however found between the overall OGR over their time in the Gave de Pau River and the  $Ba/Ca_{\text{otolith}}$  recorded during their early life (peak response). The reasons remain unclear. The main difference between the two situations was the magnitude of the change in the  $Ba/Ca_{\text{water}}$  ratio experienced by the fishes, +40% increase in the mesocosm experiment (Lapitxuri) against +120% and +400%, respectively for Medous and Sassis fry. Consequently, our data suggest that OGR cannot alone explain the high inter-individual variance of this ratio in otoliths of freshwater fish as was previously reported for

marine species (Bath et al., 2000; Walther et al., 2010; Miller, 2011; Sturrock et al., 2015). Other intrinsic factors would thus need to be considered. Future investigations using experimental approaches should examine Ba transport in blood and endolymph, and its uptake onto otolith as all these processes involves significant physiological regulation (Melancon et al., 2009; Payan et al., 2002). However, a previous study found no evidence for significant physiological control of Ba uptake into the blood of European plaice *Pleuronectes platessa* L. in a marine environment (Sturrock et al., 2014). But, unlike our study, their fishes were not exposed to a sudden and marked change in ambient Ba/Ca<sub>water</sub> ratio.

Discreet seasonal fluctuations in Ba/Ca<sub>otolith</sub>, hidden by the wide individual response (Fig. 4), seems to vary inversely to both Ba/Ca<sub>water</sub> ratio (Figure S4) and temperature (Fig. 2c). We found no evidence that Ba concentration in otoliths increases with temperature, which is in agreement with previous studies examining marine fishes (Bath et al., 2000; Fowler et al., 1995). Relative effects of temperature on otolith of a freshwater fish (*Perca flavescens*) and an estuarine species (*Acanthopagrus butcheri*) were however noticed (Collingsworth et al., 2010; Elsdon and Gillanders, 2002) but were not significant.

Changes in physiological processes that affected the composition and abundance of many organic molecules into the blood plasma over seasons may also control the amount of bioavailable Ba (Kalish, 1991; Gronkjaer, 2016; Thomas et al., 2017; Hüseyin et al., 2020) and thus the Ba/Ca<sub>otolith</sub>. A study demonstrated that Ba concentration in blood plasma exhibited limited temporal variability and was not related to changes in the protein content through time in a marine fish, suggesting a reduction in biological fractionation for this element and thus greatest potential link to environmental fluctuations (Sturrock et al., 2014). Barium was also observed to be less protein-bound than Sr in otoliths (Izzo et al., 2016). Our results also support a restricted influence of blood protein on Ba/Ca<sub>otolith</sub> during periods of stable ambient water

chemistry, although discreet seasonal oscillations with slightly higher value in autumn-winter and lower in spring-summer may be in favour of regulation by the latter.

### **Implications for environmental reconstructions**

Robust reconstruction of fish origin and movements through distinct habitats requires several fundamental assumptions: (1) otoliths record the original signal from the waters the fish lived in without major alteration; (2) any individuals living in the same location or moving between two habitats display consistent recordings (e.g. amplitude, time-lag) in their otoliths (Campana, 1999; Campana and Thorrold, 2001; Grønkjær, 2016; Walther, 2019). Although water chemistry controls the baseline level for otolith elemental ratio for a given site, our data show it is not always true. Deviations from the water chemistry are possible and potentially related to physiological effects on Sr and Ba incorporation (Walther, 2019; Marino et al., 2020).

We found that temperature and/or growth rate could induce seasonal variations in  $\text{Sr}/\text{Ca}_{\text{otolith}}$  by an order of 0.1-0.2  $\text{mmol}\cdot\text{mol}^{-1}$ . Compared to the range of variations generally observed within freshwater systems, this alteration can be considered weak between some rivers, but may impair the ability to distinguish among rivers at fine spatial resolution, especially those having close  $\text{Sr}/\text{Ca}_{\text{water}}$  signatures. Using a multi elemental approach (beyond Ba and Sr) would be more efficient for discrimination among rivers.

We showed that otolith response to a steep increase in freshwater Ba/Ca ratio resulted in a wide initial dispersal in the  $\text{Ba}/\text{Ca}_{\text{otolith}}$  ratio, implying physiological driven factors could largely outweigh the environmental signal in some individuals and thus should be considered while assessing lifetime movements from high-resolution Ba chemical profile. Furthermore, the time-lag to reach a new equilibrium when fish moved among habitats, highly differs according to individuals. Consequently, brief incursions in habitats having Ba-enriched levels might be thus challenging to assess for sure. Determining accurate habitat fingerprints with

645 enough confidence will thus require selecting a suitable portion of the otolith on each individual  
646 in order to sample exclusively the environmental signal and remove noise associated with  
647 physiological processes.

## 649 **Conclusions**

650 Our findings show that Sr/Ca and Ba/Ca ratios in freshwater systems control the baseline level  
651 for their otolith elemental ratios at a given site. However, variations through time would still be  
652 either controlled by extrinsic (temperature) and/or intrinsic factors (growth rate, metabolism)  
653 and/or changes in water chemistry (i.e., flood events). Opposite Sr/Ca fluctuations between  
654 otolith and water chemistry suggest that seasonal changes in physiology (growth, blood  
655 complexing capacity) and temperature affect the otolith signal by adding noise to the global  
656 background driven by the water chemistry. Otolith Ba/Ca ratio can also largely deviate from  
657 the water-predicted ratios mostly at transitions following abrupt increases in the water ratio for  
658 resident fish (flood event) or for fish changing habitats (movement between streams). Finally,  
659 wider inter-individual variability in the Ba/Ca otolith response was observed compared to  
660 Sr/Ca, either during periods of ambient water stability or environmental changes, reducing its  
661 discriminating power in otolith freshwater application. Strontium and barium have been the two  
662 pillars of the research analyzing otoliths, undoubtedly major breakthroughs were made by  
663 measuring Sr/Ca and Ba/Ca ratios but they also display some limitations and the community  
664 should incorporate additional elements, specifically for freshwater systems.

665  
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## Figures

Fig. 1.  $\text{Sr}/\text{Ca}_{\text{water}}$  temporal variability (black squares) and  $\text{Sr}/\text{Ca}_{\text{otolith}}$  profiles of *Salmo salar* parr of age-0<sup>+</sup> (continuous thin black lines) caught in September 2009 (green) and that experienced steep decrease in  $\text{Sr}/\text{Ca}_{\text{water}}$ . Predicted  $\text{Sr}/\text{Ca}_{\text{otolith}}$  ratio (average: thick blue line; minima and maxima: dotted blue lines) assumes that otolith variations through time rely exclusively on changes in the ambient chemistry. Vertical lines delimit the successive habitats occupied by fish since eggs deposition.

Fig. 2. Results for parr that did not experience significant change in  $\text{Sr}/\text{Ca}_{\text{water}}$ . Panel a (top):  $\text{Sr}/\text{Ca}_{\text{water}}$  temporal variability (black squares) and  $\text{Sr}/\text{Ca}_{\text{otolith}}$  profiles of *Salmo salar* parr of age-0<sup>+</sup> (continuous thin lines: black=2006, green=2009 and orange=2010). Panel b (intermediate):  $\text{Sr}/\text{Ca}_{\text{otolith}}$  profiles of *Salmo salar* parr of age-1<sup>+</sup> during their stay in the Gave de Pau River and collected in September 2006 (each colour represents an individual). Both panels: Predicted  $\text{Sr}/\text{Ca}_{\text{otolith}}$  ratios (average: thick blue line; minima and maxima: dotted blue lines) assumes that otolith variations through time rely exclusively on changes in the ambient chemistry. Vertical lines in panel a) delimit the successive habitats occupied by age-0<sup>+</sup> fish collected in autumn. Vertical dotted line in panel b) delimits grow years 2005 and 2006. Panel c) seasonal temperature (°C) variability.

Fig. 3.  $\text{Ba}/\text{Ca}_{\text{water}}$  temporal variability (black squares) and  $\text{Ba}/\text{Ca}_{\text{otolith}}$  profiles of *Salmo salar* parr of age-0<sup>+</sup> (continuous thin lines: black=2006, green=2009 and orange=2010) that were exposed to significant increase in  $\text{Ba}/\text{Ca}_{\text{water}}$  ratio following their stocking. Panel a (top) and b (bottom) show data for parr reared in the Medous and Sassis hatcheries, respectively. Predicted  $\text{Ba}/\text{Ca}_{\text{otolith}}$  ratio (average: thick blue line; minima and maxima: dotted blue lines) assumes that



otolith variations through time rely exclusively on changes in the ambient chemistry. Vertical lines delimit the successive habitats occupied by fish since eggs deposition.

Fig. 4. Results for age-1<sup>+</sup> parr caught in 2006 that experience slight seasonal fluctuations in  $Ba/Ca_{\text{water}}$  (Gave de Pau River). Each colour represents an individual as in figure 2b. Predicted  $Ba/Ca_{\text{otolith}}$  ratios (average: thick blue line; minima and maxima: dotted blue lines) assuming that otolith variations through time rely exclusively on changes in the ambient chemistry. Vertical dotted line delimits year 2005 and 2006.

Fig. 5. Otolith response to a three weeks pulse in the  $Ba/Ca_{\text{water}}$  ratio during juvenile growth under natural conditions in the Lapitxuri field-station. Green, orange and blue refer to [35-45], [50-55] and [60-75] range sizes (mm), respectively. Predicted  $Ba/Ca_{\text{otolith}}$  ratio (average: thick black line; minima and maxima: dotted black lines) assumes that otolith variations through time rely exclusively on changes in the ambient chemistry. Vertical dotted line at day one hundred and ten corresponds to the second alizarine redS marks (AM2). The shaded area corresponds to the period of 40% increase of water  $Ba/Ca$  ratio under the effect of the flood event.

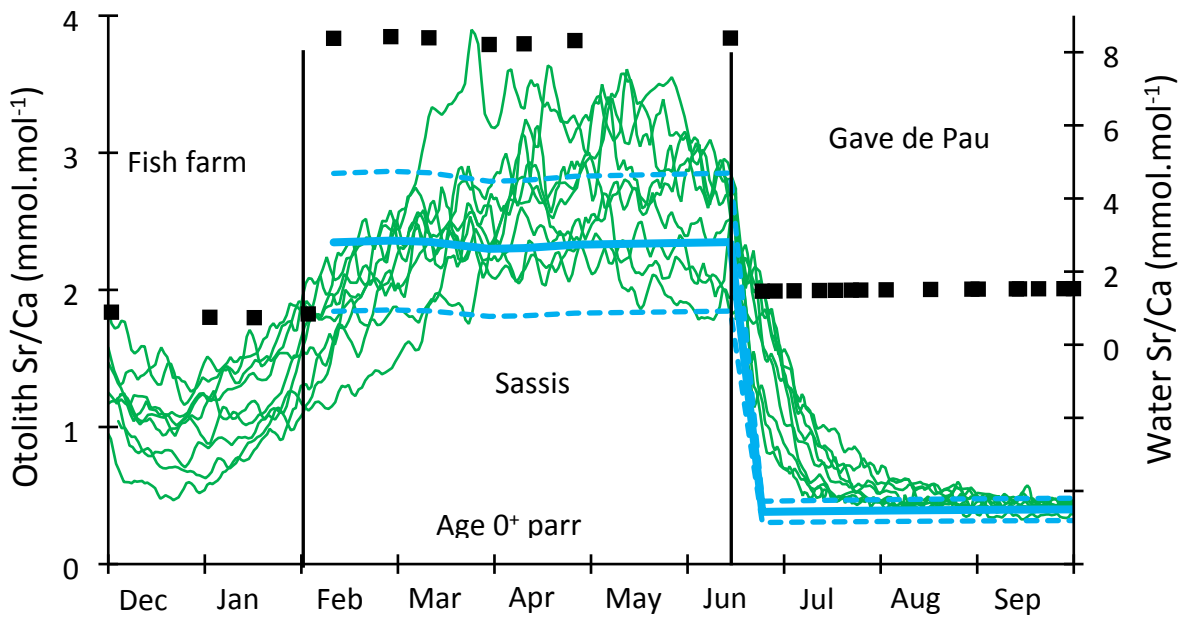
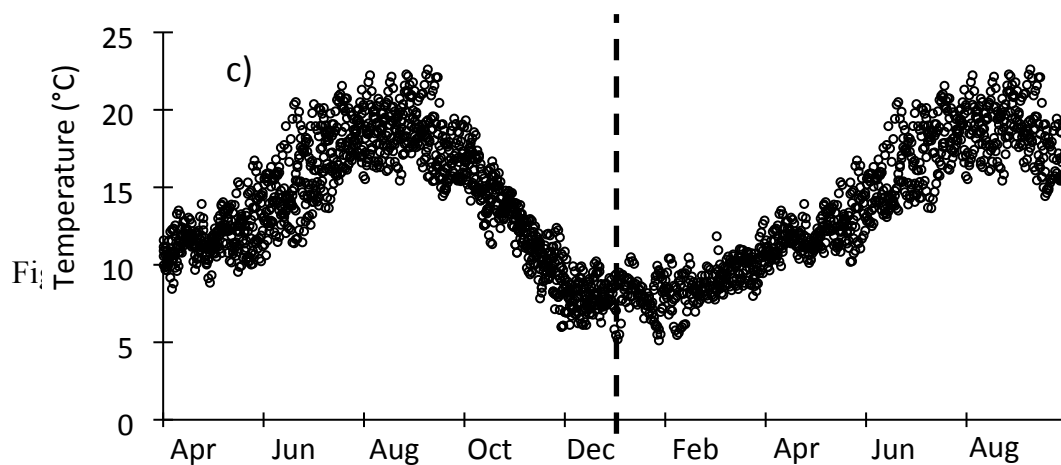
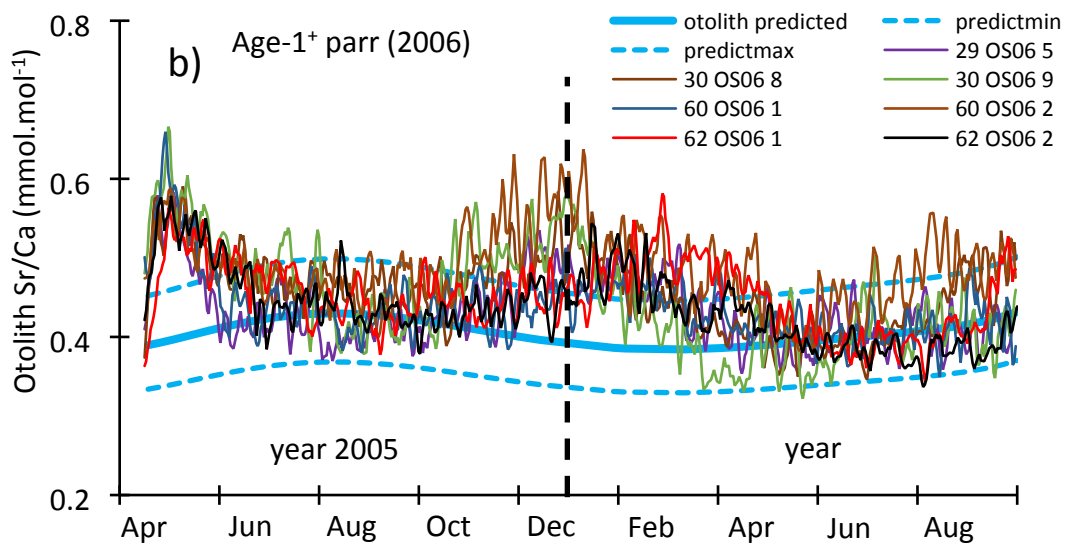
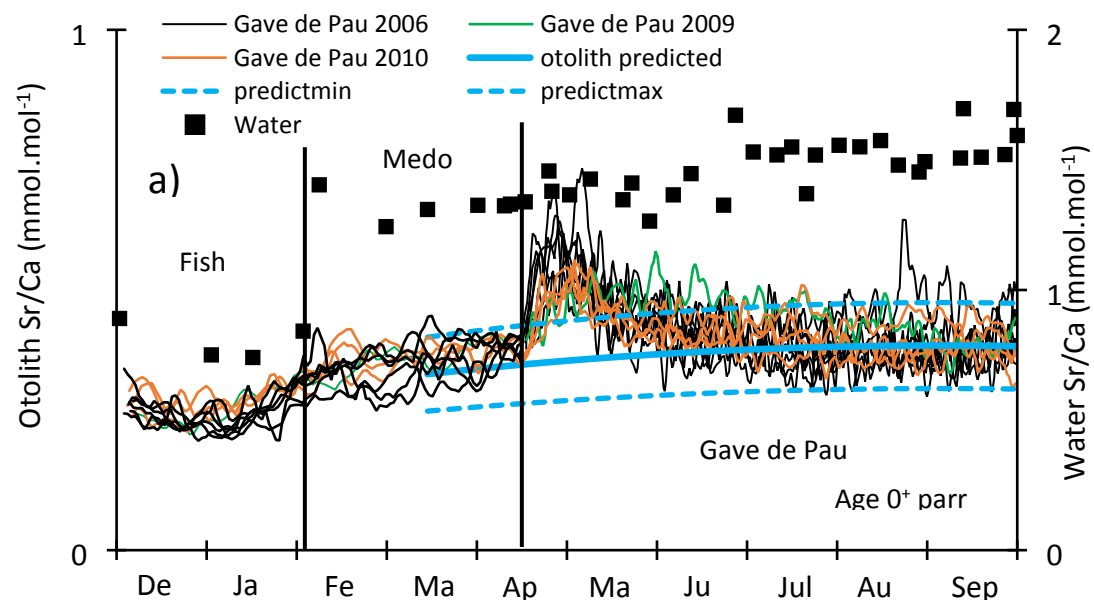


Fig. 1.



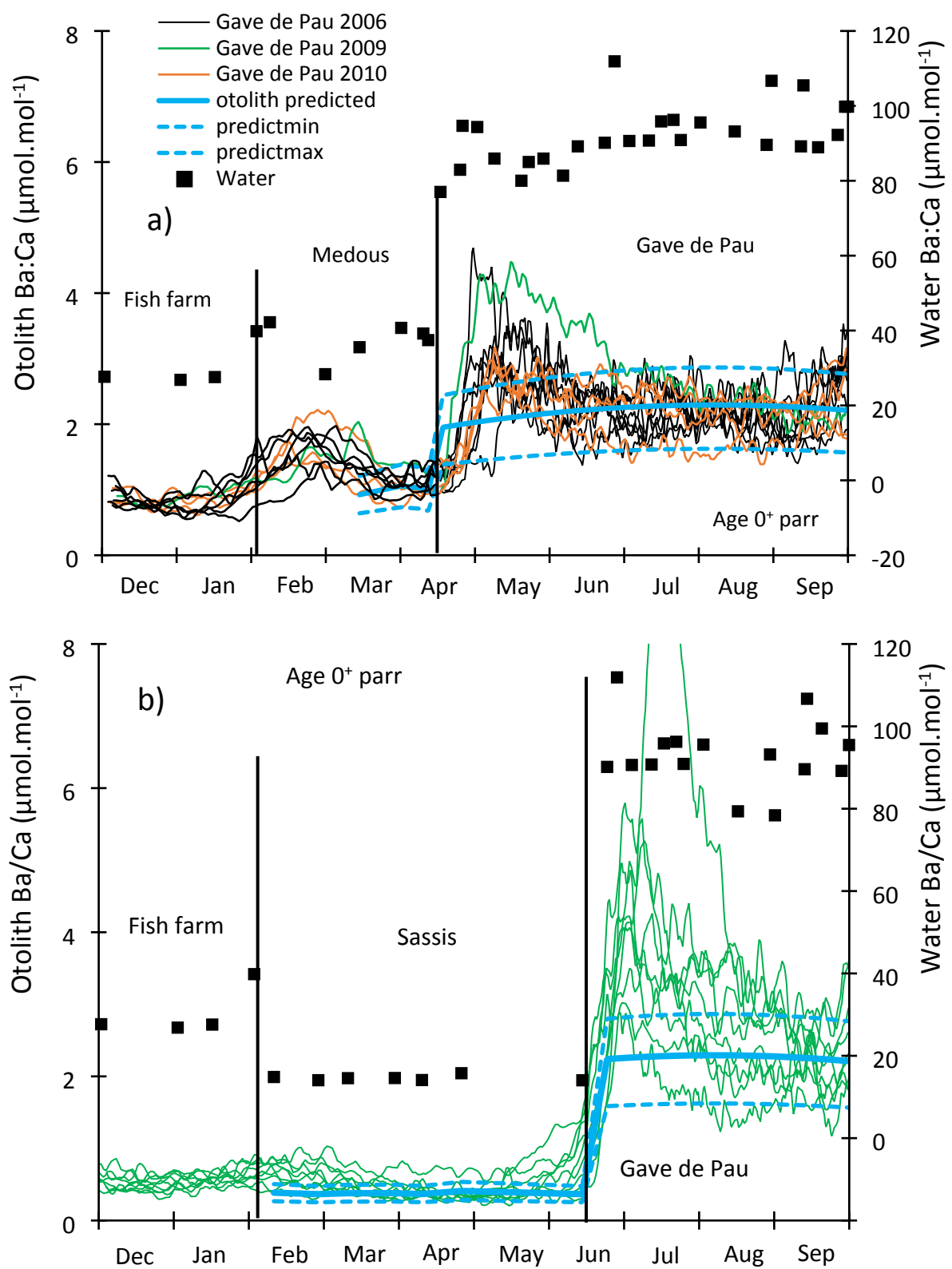


Fig. 3.

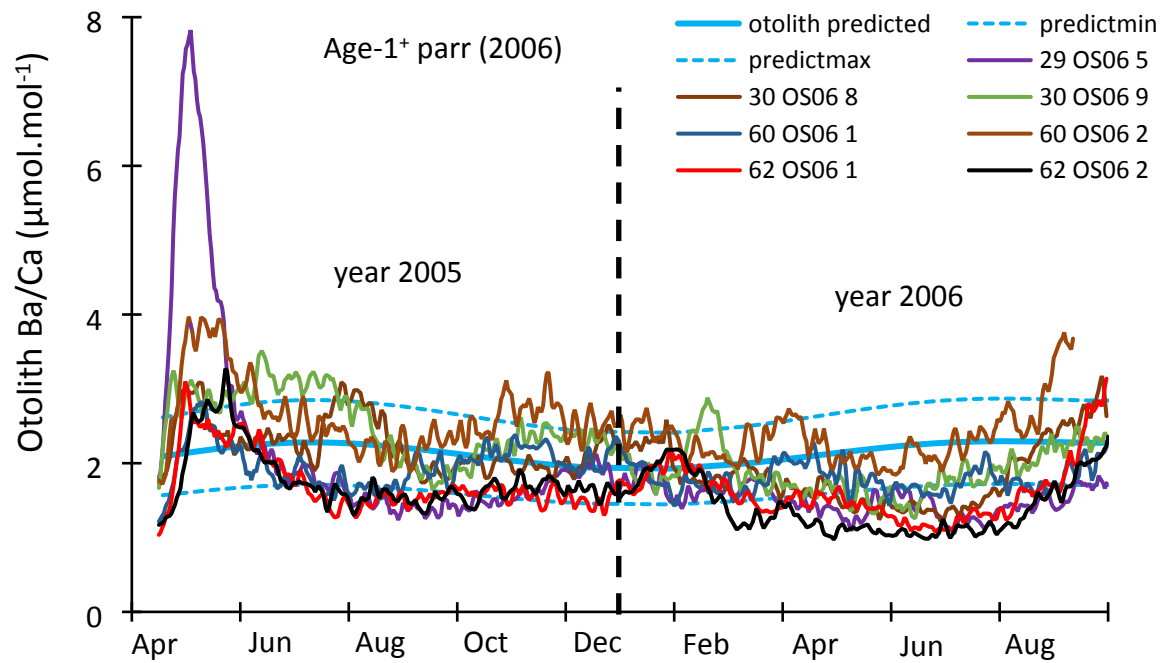


Fig. 4.

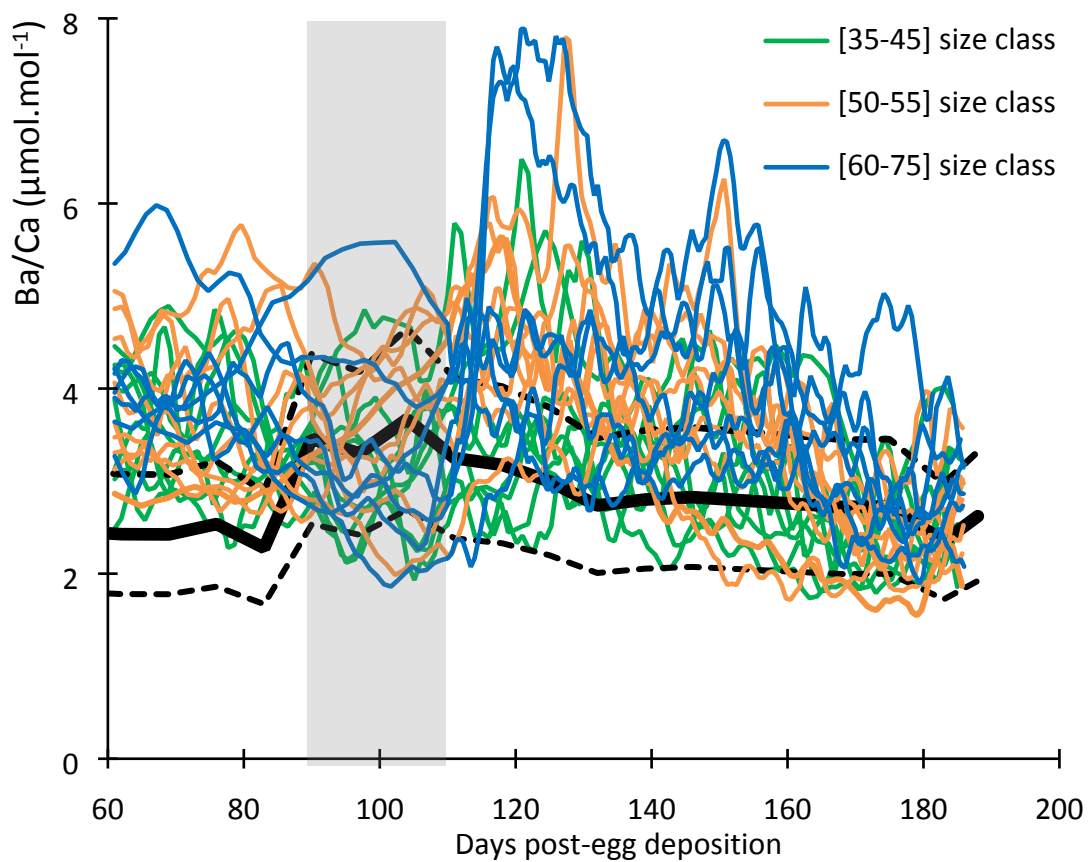


Fig. 5.

1 Table 1. Chemical scenarios studied and fish data (sampling year, number, age, site of collection) are presented for the Sr/Ca and Ba/Ca elemental  
2 ratios. Aims of the work and statistical analyses used are also reported in the last two column.

3  
4 Table 2. Average and standard deviation of Sr/Ca (mmol.mol<sup>-1</sup>) and Ba/Ca (μmol.mol<sup>-1</sup>) ratios in otoliths and waters from 13 rivers of the Adour  
5 basin taken from a previous study (Martin et al., 2013a), from the Lapitxuri facility and from the Medous and Sassis hatcheries. For each river,  
6 ratios correspond to the average of the last 100 μm of otolith accretion of n fish collected in September (Adour) and June (Lapitxuri) and n water  
7 samples collected during the end of the summer period (August to September months) and May month (Lapitxuri). The average of D<sub>Sr</sub> from all the  
8 rivers as well as the three average D<sub>Ba</sub> from three concentrations range, used in this study to predict otolith Sr/Ca and Ba/Ca ratios from the water  
9 chemistry are also given.

10

11 Table 3. Ba/Ca<sub>otolith</sub> (mean, SD, CV) and otolith growth rate (OGR) for three time periods (overall stay in the Lapitxuri channel, flood event, normal  
12 flow period at the end of the experiment) according to the three size classes investigated. Different letters A, B, a, b and c refer to significant  
13 difference between size classes at the 0.05 confidence level. Statistical significance in linear relationships between Ba/Ca<sub>otolith</sub> and OGR are also  
14 provided.

15

16 Table 4: Total deviance (%) explained by the full model and partial deviance explained by each explanatory variable for each case study using a  
17 Generalized-Additive-Mixed-Models (GAMM). Water-based otolith composition (mean-value only, non-linear trend), water temperature (non-  
18 linear trend) and individual Fulton's index (linear trend) were considered as variables treated as fixed effect and 'individuals' as variable treated as  
19 random effect.

20

21

22 Table 1

Elemental ratio	Year of capture (n)	Fish Age	Treatment / Scenario	Aims of the work	Statistical tests	
Sr/Ca	2009 (n=9)	0 <sup>+</sup>	Sassis to Gave de Pau (mid-June): steep decrease in water Sr/Ca	i) otolith Sr/Ca and Ba/Ca ratios reliably reflect life-history scenarios?	<b>Generalized-Additive-Mixed-Model (GAMM):</b>  ∝ <i>water-based prediction of otolith composition; water temperature and individual Fulton's index</i> as fixed effect ∝ <i>'individuals'</i> as random effect	
	2006 (n=8)	0 <sup>+</sup>	Medous to Gave de Pau (mid-April): sligth increase in water Sr/Ca			
	2009 (n=1)					
	2010 (n=4)					
Ba/Ca	2006 (n=7)	1 <sup>+</sup>	Medous to Gave de Pau (mid-April): sligth seasonal fluctuations in water Sr/Ca	ii) temperature, fish condition factor and individual variability explain/influence deviations between observed and water predicted ratios?	<b>Pearson or Spearman correlation tests:</b> Otolith growth rate (OGR) vs interindividual variability	
	2009 (n=9)	0 <sup>+</sup>	Sassis to Gave de Pau (mid-June): six fold increase in water Ba/Ca			
	2006 (n=8)	0 <sup>+</sup>	Medous to Gave de Pau (mid-April) : three fold increase in water Ba/Ca			
	2009 (n=1)					
	2010 (n=4)					
	2006 (n=7)	1 <sup>+</sup>	Medous to Gave de Pau (mid-April) : three fold increase + slight seasonal fluctuations in water Ba/Ca			
	2011	6 months	Lapitxuri experiment: transient increase in water Ba/Ca			
	[35-45 mm]: n=8					
[50-55 mm]: n=9						
[60-75 mm]: n=7						

23



24 Table 2

25

	Otolith Sr/Ca (mmol.mol <sup>-1</sup> )			Water Sr/Ca (mmol.mol <sup>-1</sup> )			D <sub>Sr</sub>	ΔD <sub>Sr</sub>	Otolith Ba/Ca (μmol.mol <sup>-1</sup> )			Water Ba/Ca (μmol.mol <sup>-1</sup> )			D <sub>Ba</sub>	ΔD <sub>Ba</sub>		
	<i>n</i>	<i>mean</i>	SD	<i>n</i>	<i>mean</i>	SD			<i>n</i>	<i>mean</i>	SD	<i>n</i>	<i>mean</i>	SD				
<i>Rivers</i>																		
Lourdios	12	0.71	0.04	4	2.76	0.65	0.26	0.08	12	0.91	0.16	4	39.4	6.6	0.023	0.008		
Verts	10	0.66	0.07	8	2.39	0.18	0.28	0.05	10	1.02	0.22	8	40.9	2.7	0.025	0.007		
Ossau	27	0.52	0.05	11	1.83	0.15	0.28	0.05	27	1.13	0.24	11	40.9	4.2	0.028	0.009		
Aspe	14	0.95	0.05	6	4.01	0.45	0.24	0.04	14	1.15	0.24	6	42.9	4.4	0.027	0.008		
Laurhibar	9	0.78	0.08	6	3.35	0.18	0.23	0.04	9	1.27	0.19	6	49.5	7.8	0.026	0.008		
Oloron	22	0.76	0.11	14	2.63	0.34	0.29	0.08	22	1.52	0.42	14	51.9	6.5	0.029	0.012		
Saison	13	0.75	0.06	12	2.64	0.28	0.28	0.05	13	1.70	0.35	12	60.3	5.5	0.028	0.008		
Ouzom	16	0.42	0.04	4	1.76	0.11	0.24	0.04	16	1.71	0.42	4	75.7	1.2	0.023	0.006		
Downstream																		
Pau	13	0.40	0.04	8	1.58	0.18	0.25	0.06	13	2.26	0.39	8	95.5	8.7	0.024	0.006		
Béhérobie	17	0.46	0.03	6	1.65	0.25	0.28	0.06	17	2.51	0.35	6	104.7	14.3	0.024	0.007		
Upstream Pau	4	0.41	0.03	7	1.69	0.08	0.24	0.03	4	2.58	0.24	7	132.3	10.5	0.019	0.003		
Arnéguy	17	0.29	0.04	5	1.15	0.17	0.25	0.07	17	4.34	0.93	5	253.4	52.7	0.017	0.007		
Lapitxuri	24	0.36	0.04	6	1.22	0.01	0.30	0.04	24	2.62	0.57	6	171	13	0.015	0.004		
							Average											
							D <sub>Sr</sub>	<b>0.26</b>	<b>0.05</b>	Water Ba/Ca <50μmol.mol <sup>-1</sup>							<b>0.026</b>	<b>0.008</b>
									Water Ba/Ca 80-120 μmol.mol <sup>-1</sup>							<b>0.024</b>	<b>0.006</b>	
									Water Ba/Ca >120 μmol.mol <sup>-1</sup>							<b>0.017</b>	<b>0.005</b>	
<i>Hatcheries</i>																		
Medous	10	0.39	0.05	4	1.27	0.16	-	-	10	1.19	0.30	4	36.7	6.3	-	-		
Sassis	9	2.60	0.44	7	8.34	0.08	-	-	9	0.44	0.14	7	14.6	0.6	-	-		

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Table 3

Life period	Size class (mm)	Otolith Ba/Ca (μmol.mol <sup>-1</sup> )					OGR (μm.day <sup>-1</sup> )				Ba/Ca vs OGR (α)
		Mean	SD	CV (%)	predicted§	Mean	SD	CV (%)			
AM2-edge (110-187)	[35-45]	3.08	0.54	A	18	2.99±0.45	1.62	0.13	A	8	<i>r</i> = 0.66
AM2-edge (110-187)	[50-55]	3.52	0.52	B	15		1.95	0.14	B	7	<i>p</i> = 0.006
AM2-edge (110-187)	[60-75]	3.9	0.83	B	21		2.09	0.16	B	8	
Flood event (110-135)	[35-45]	3.52	0.54	a	15	3.49±0.20	1.54	0.16	a	10	<i>r</i> = 0.74
Flood event (110-135)	[50-55]	4.44	0.59	b	13		1.79	0.24	b	14	<i>p</i> = 0.001
Flood event (110-135)	[60-75]	4.64	0.97	b	21		1.81	0.24	b	13	
Normal flow (168-187)	[35-45]	2.48	0.43	c	17	2.66±0.20	1.85	0.23	c	12	<i>r</i> = 0.32
Normal flow (168-187)	[50-55]	2.46	0.43	c	17		1.90	0.22	c	12	<i>p</i> = 0.24
Normal flow (168-187)	[60-75]	2.73	0.42	c	15		1.96	0.33	c	17	

$\alpha$  otolith growth rate estimated as the distance between anchor points divided by the number of days between them (AM2 to edge)

§ mean daily growth rates over the defined period of time

§ otolith Ba:Ca ( $\mu\text{mol.mol}^{-1}$ ) predicted from water chemistry

Table 4

Elemental ratio	Treatment	Chemical scenarios	Fish Age	Total explained deviance (%)	Partial deviance (%)			
				Full model	Water-based otolith composition	T (°C)	Fulton's index	Interindividual variations
Sr/Ca	Sassis to Gave de Pau (mid-June)	Steep decrease (five fold)	0 <sup>+</sup>	97.6	4.48	3.67	1.62 10 <sup>-4</sup>	6.42
	Medous to Gave de Pau (mid-April)	Sligh increase (7%)	0 <sup>+</sup>	66.1	10.93	15.17	4.94 10 <sup>-6</sup>	20.89
		Sligh seasonal oscillations (7%)	1 <sup>+</sup>	42	18.76	9.43	1.33 10 <sup>-5</sup>	18.54
Ba/Ca	Sassis to Gave de Pau (mid-June)	Six fold increase	0 <sup>+</sup>	92	24.34	4.34	4.71 10 <sup>-5</sup>	23.2
	Medous to Gave de Pau (mid-April)	Three fold increase	0 <sup>+</sup>	71.9	6.42	22.94	2.25 10 <sup>-3</sup>	23.35
		Three fold increase + sligh seasonal oscillations (15%)	1 <sup>+</sup>	28.5	8.88	4.77	1.75 10 <sup>-4</sup>	21.09
	Lapitxuri experiment	Transient increase	6 months	79.9	23.4	2.96	1.56 10 <sup>-4</sup>	51.36