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Life history of the Small sandeel, *A. tobianus*, infered from otolith microchemistry. A methodological approach.

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Abstract

Knowledge of life history and connectivity between essential ecological habitats are relevant for conservation and management of species and some natural tracers could be used to study the lifecycles of small or short-lived marine fishes. Although sandeels are central in marine food webs and are key species, there is incomplete knowledge about population mixing and migration patterns. For the first time the use of the otolith microchemistry on sandeel species is evaluated in the case of the Small Sandeel. Variations in microchemical fingerprints of 13 trace elements are performed with a Femtosecond LA-ICPM from the core to the margin of sagittal otolith and are compared within and between otoliths extracted from 34 fishes sampled in three different sites along the coast of the south-western English Channel in France. Firstly, preliminary investigations on the validity of the method revealed that Mg/Ca was the only ratio significantly dependant on fish ontogeny and sampling season. Secondly, the Mn/Ca, Zn/Ca, and Cu/Ca ratios enabled us to significantly discriminate among sampling sites. Thirdly, microchemical fingerprints of each life stage varied significantly among sampling sites but not within them, suggesting high site fidelity over relatively short distances. Finally, the fingerprints of all life stages were significantly different from those of the larval and metamorphosis stages. The otolith microchemistry could detect change of signature relative to the shift from a pelagic behaviour to a resident bentho-pelagic behaviour during the middle of the juvenile stage in Small Sandeels. Hence, analysis of trace element fingerprints in otoliths appears to be a valuable method to further studies on ontogenic habitat change, population mixing and variation of life history and be helpful for the management at local or regional scales of short-lived species such as those belonging to other Ammodytidae.

Keywords: Ammodytidae, short lifespan, multi-element signature, habitat use, intertidal beaches, biological tracer

1. Introduction

Identification of connectivity between essential habitats during the lifespan of species is key to the population ecology and management of fish. This has been studied in numerous marine fishes that move from estuaries or salt marsh systems during their juvenile stage to offshore habitats for growth and/or spawning (Hansen and Quinn, 1998; Fritsch, 2005; Aarestrup et al., 2009, Daverat et al., 2011; Mercier et al., 2012). Movements and migrations are also commonly studied in diadromous fish to study their land-ocean connectivity (Koutsikopoulos et al., 1995; Feutry et al., 2011; Bultel et al., 2014). However, such connectivity has most often been studied for large fish with long lifespans (Galuardi and Lutcavage, 2012) and with migration loops by fish across large distances (Secor and Zdanowicz, 1988; Block et al., 2001) and/or between contrasting habitats (e.g. marine to inland, estuarine to marine, etc.) (Milton and Chenery, 2003; Daverat et al., 2011; Isnard et al., 2015).

The focus on short, holobiotic migration loops, for example in coastal areas and for small fish with short lifespans is increasing (Goto and Arai, 2003; Keith, 2003; Aldanondo et al., 2010; Tabouret at al., 2011). This is relevant not only from a fundamental perspective, but also from a management perspective. For example, many small coastal fishes, such as sandeels (Ammodytidae family), are keystone species of marine ecosystems. Their mid-trophic position in the foodweb make them a forage prey for top predators, including marine mammals, seabirds, and fish species (Wanless et al., 2005; Eliasen et al., 2011; Engelhard et al., 2013, 2014). Sandeels are also economically important, but their stocks appear to fluctuate and have declined in recent years through over-fishing or because of global change, which has modified the structure of marine foodwebs (Wanless et al., 2004; Frederiksen et al., 2007, 2011). In recent years, some studies on sandeel communities were conducted to examine the impact caused by the development of offshore wind farms and marine sediment extraction for construction on these fishes (e.g. van Deurs et al., 2012). Indeed, well-oxygenated sandbanks, preferably with a low fraction of silt and clay (Wright et al., 2000), play important ecological roles for sandeels, which exhibit the unusual habit of alternating between pelagic swimming for feeding and lying buried in the sand substrate even at low tide in intertidal areas, at night and during winter (Winslade, 1974; Robards et al., 2000; Jensen et al., 2011; van Deurs et al., 2011). Sandbanks could consequently be considered an essential ecological habitat (EEH) (e.g. spawning ground, nursery, feeding, or resting habitat) (Rijnsdorp et al., 2009; Petigas et al., 2013) for sandeel.

The Small Sandeel, *Ammodytes tobianus*, the most abundant sandeel species in intertidal sandy habitats, has a maximum age of 7 years old, can reach maturity at one year old and spawns twice a year (spring and autumn) (Reay, 1973, Kopp, 1979; O'Connel and Fives, 1995), and it also remains unclear whether the Small Sandeel is an obligate intertidal spawner (Robards et al., 1999). The Small Sandeels are rarely caught offshore (Jensen et al., 2004; van Deurs et al., 2011). Despite the ecological and commercial importance of sandeels (Engelhard et al., 2014), studies have examined mainly the Lesser Sandeel, *Ammodytes marinus*, in the North Sea (Wright, 1993; Wright and Bailey, 1996; Wright et al., 2000; Frederiksen et al., 2011). Recent investigations on this species have shown very

limited migration behaviour with a high site fidelity to a 'home sandbank' after settlement and little mixing between grounds (Engelhard et al., 2008; Jensen et al., 2011). Furthermore, for the Lesser Sandeel, dispersion between sandy areas is suspected to occur via the drift of pelagic larvae controlled by ocean currents (Christensen et al., 2008). The study of the Lesser Sandeel movements between sandbanks has been tested in through field studies and models (Christensen et al., 2008; Engelhard et al., 2008; Jensen et al., 2011) but the mixing between populations and its life histories are not totally unravel.

As it is not currently possible to perform telemetry studies with small fish species, the purpose of the present paper is to validate, for the first time for an Ammodytidae species, the Small Sandeel, the use of otolith microchemistry as a potential tracer of movements between habitats during the life history, which may exhibit little migration behaviour, except maybe during its larval stage. Otoliths are calcified inert structures located in the inner ear of fish that grow continuously throughout life (Campana and Neilson, 1985). Because they incorporate the chemical elements of the surrounding waters at the time of deposition, otoliths act as natural tags (Milton and Chenery, 2001; Lin et al., 2007; Marohn et al., 2009), and their structures (i.e. macrostructure and microstructure) as recorder of life stages (Campana, 1999). Furthermore, otolith microchemistry also provides powerful information on migration patterns and the habitat used during their life cycle (Gillanders, 2005; Arai and Hirata, 2006; Feutry et al., 2011; Lord et al., 2011; Mercier et al., 2012).

Our hypotheses are: 1) if the Small Sandeel exhibit comparable limited migration behaviour to the Lesser Sandeel, then their otoliths will have distinct microchemical fingerprints between individuals from different sandy beaches; and 2) if sandeels do not use different habitats during their lifecycle, reflecting a resident behaviour, the elemental composition will not vary within the otolith from the core to the margin.

This study firstly assesses the variation in size class distribution of Small Sandeels over a one-year bimonthly survey to detect recruitment periods and growth rates. The use of the otolith macrostructure is validated by comparing the growth calculated from cohorts and from otoliths. Then, hypotheses are tested by comparing the elemental composition within otolith macrostructure and between otoliths of Small Sandeels captured in three nearby coastal areas of Northern Brittany and Normandy, France. Finally, to validate the first use of otolith microchemistry on sandeel species, the stability of the microchemical signatures among seasons and ontogenic development are tested. The results are used to discuss whether microchemical tracers of the otoliths are useful to analyse the life history traits of a coastal, non-migratory, short-lived fish species such as the Ammodytidaes.

2. Materials and methods

2.1. Study area and fish sampling

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The three studied intertidal sandbanks are located in the south-west English Channel along the coast of the Norman-Breton Gulf. The main study site, Lancieux Bay, is located at the mouth of the Frémur estuary, and the other sites chosen for testing the site fidelity of Small Sandeels were Rotheneuf Bay and at the Chausey archipelago (respectively 20 and 40 km from Lancieux) (Figure 1). Sandeels were sampled at Lancieux Bay with a shovel in the sand ripple marks of the beach at low tide twice a month for a year (February 2012 to January 2013). All of the fish were stored at -20°C within one hour of capture for further identification and measurement (fork length (FL) in mm) at the laboratory.

In order to test ontogenic and seasonal variations in microchemical signature, 24 sagittal otoliths were extracted from juveniles and adults and in different seasons: 5 0-group juveniles caught in February and July, and 5 adults caught in July and 9 in November. Additionally, to identify spatial signatures among the three sites, sagittal otoliths were extracted in July, from 2 0-group juveniles and 3 adults caught at Chausey archipelago and 5 0-group juveniles at Rotheneuf Bay.

2.2. Otolith preparation and analysis

After extraction, the sagittal otoliths were washed three times in an ultra-pure water bath (milliQ 0.0055 µS). After the remaining tissues were removed under a binocular microscope, otoliths were dried and stored in 1.5-mL plastic Eppendorf tubes. The left otolith extracted from each fish was embedded in araldite resin 2020 (Huntsman) with the sulcus acusticus downward. They were grounded in the sagittal plane up to the core with ultra-pure water and sandpaper with grains gradually decreasing from 2400 µm to 1200 µm, 9 µm, and 3 µm. Finally, the otoliths were rinsed with ultra-pure water and air-dried.

Otolith microchemical composition was assessed using 257 nm femtosecond laser ablation (Lambda 3, Nexeya, France) inductively coupled with plasma mass spectrometry (Elan DRCII, Perkin Elmer) (LA-ICPMS). This delivers 360 fs pulses at wavelengths of 1030 nm and can be operated at high repetition rates (up to 100 kHz). A 2D galvanometric scanner allows the fast movement of the laser beam (10 µm) at the surface of the sample to simulate virtual beam shaping when the laser is operated at a high repetition rate. Considering the otolith growth ring pattern, an elongated laser beam (10×50 μm^2) was simulated in order to preserve the high spatial resolution while keeping the highest signal sensitivity. The laser was operated at 300 Hz with a pulse energy of 35 µJ while the scanner was doing a permanent 35-µm-wide, oscillating movement at a speed of 2 mm/s, resulting in a 20×50 µm laser beam. Combined with this oscillating movement, the sample was continuously moved along the posterior axis from the nucleus to the edge of the otolith at a speed of 5 µm/s, resulting in an uninterrupted ablation on the grounded surface. In order to prevent a blast effect on the nucleus, the ablation was started 200 µm before the nucleus. The ablation depth was evaluated at 10 µm.

At the beginning and end of each session, careful calibrations were carried out using NIST 610, 612, and 614 (National Institute of Standards and Technology). Quality control was systematically

evaluated using pelletized CRM NIES 22 otolith powder (Certified Reference Material produced by the National Institute for Environmental Studies). ^{43}Ca was used as an internal standard for each ablation to correct for instrumental error in terms of ablation yield, sample transport and detection. Analysed isotopes were ^{86}Sr, ^{135}Ba, ^{138}Ba, ^{24}Mg, ^{26}Mg, ^{55}Mn, ^{63}Cu, ^{65}Cu, ^{66}Zn, ^{68}Zn, ^{57}Fe, ^{232}Th, and ^{238}U, which are frequently used in microchemistry studies (Vasconcelos et al., 2011). Isotopes for which 75% of the measurements were above the limit of detection for at least one individual were retained. Furthermore, for elements with two isotopes (e.g. ^{63}Cu, ^{65}Cu), only the isotope with the highest natural abundance was kept after checking that no spectral interference was affecting the reliability of the result. After standardization by calcium (Campana, 1999), the remaining element ratios were Ba/Ca, Sr/Ca, Mn/Ca, Zn/Ca, Cu/Ca, Fe/Ca, and Mg/Ca.

To match the microchemical signatures with the different life stages of sandeels, macrostructural analyses of the otoliths (Figure 2) were performed. The larval stage (L) was clearly distinct on the otolith with a change in the growth axis, which corresponds to settlement into sediment (Wright, 1993). Opaque zones (under transmitted light) correspond to rapid growth in summer (S), whereas translucent zones were interpreted as low growth periods in winter (W) (ICES, 1995, 2006). Finally, we postulated that fish had spent sufficient time at the site of capture to assimilate a local fingerprint. The site of capture signature (C) was therefore considered to be the fingerprint measure within the external part of the otolith (Number 9, Figure 2B). After the continuous ablation, each otolith was photographed to measure larval, translucent, and opaque zones from the core to the end of otolith. The average of all of the element ratios was assessed at the centre of each zone except for the first summer growth zone (S0). This larger summer growth zone was divided into three sub-zones where means of element ratios were calculated (i.e. at the beginning (S0b, 20 µm after the larval stage), middle (S0m), and end (S0e) of the S0 zone) (Figure 2B). The length of the ablation segment used to calculate the mean element ratios was adjusted to the width of each zone, which varied among fish and according to their location within the otolith. When the identified opaque or translucent zone measured more than 200 µm, the mean of element ratios was calculated over a 100 µm distance, and when it was inferior to 200 µm, the mean was calculated over a 50 µm distance. Finally, the signature of capture was calculated over 30 µm at 20 µm from the edge of the otolith.

2.3. Data analysis

At Lancieux Bay, sampling was twice a month and a total of 642 *Ammodytes tobianus* were measured. However, for simplification, the size-class distributions were plotted on a monthly basis. Following a cohort analysis, age classes were determined and enabled estimation of the season of birth of juveniles. A code, for example G0 A2011, was attributed to each individual and represents the cohort (i.e. age class: G0, G1, etc.), the season (S: spring; A: autumn) and the year of birth. Age estimations were validated using age readings of otoliths that were extracted for the microchemistry and macro-

structural analyses (n = 24). The growth at one year of age was only calculated for two young cohorts (G1 and G0 A2011), for which it was possible to estimate the season of birth.

A linear model (LM) between the fork length (FL) of fish and the total length of their otolith (n = 44) with a Pearson correlation test (the normality and homoscedasticity of data were verified by Shapiro-Wilk and Bartlett tests) was performed to validate that otolith length was a proxy for fish length. This relationship permitted us to back-calculate small sandeel growth and to link different life stages of fish to LA-ICPMS results. The length of the larval stage and the first growth zone in otoliths (n = 24) were compared between the different cohorts using a linear model (LM).

Secondly, to validate the value of otolith microchemistry in Ammodytidae, the potential ontogenic and seasonal effects were analysed with linear model (LM) on the microchemical fingerprint from the marginal zone (i.e site of capture (C), see Figure 2B) by comparing juveniles and adults sampled at different times (juveniles caught in February and July and adults in July and November).

For all LM the normality of residuals was verified with the QQ-plot and there was no violation of the assumption to apply the Gaussian distribution. When a significant difference was detected with the Anova (F-test) type III (for the un-balanced data ('car' R package)), a multiple pair-wise comparison (Tukey post-hoc test) was applied ('multcomp' R package). For the highly unbalanced data, which occurred when analysing the otolith widths of the larval stage and the first growth zone, a bootstrap with 1000 iterations was applied after the Anova type III to verify the robustness of the results.

Finally, to distinguish the potential difference in habitat fingerprints on otoliths, the fingerprints of the marginal zone (C) of adults and juveniles captured at the three sites were compared (juveniles and adults from Lancieux and Chausey, only juveniles from Rotheneuf). Three different classification methods were used to estimate the contribution of elements measured in otoliths since some elements could provide more noise than real signal: linear discriminant analysis (LDA) and two learning methods, random forest (RF) and artificial neural networks (ANN), which are less demanding in terms of assumptions than LDA. For the LDA, capture fingerprint data met requirements for normality and homoscedasticity (i.e verified by Shapiro-Wilk and Bartlett tests), and all element ratios were standardized to give them the same weight due to differences in magnitude (Mercier et al., 2011). RF, is a tree classification method, which separates at each node the dataset in binary groups. This enables to randomly look for the group of element ratios that maximizes the homogeneity into the two groups. Each group then splits again following the same procedure until no more homogeneity is found. ANN is a system of interconnected neurons, which computes values from input neurons to hidden and finally to output neurons and linked by a function (linear, logarithmic, etc.). To identify the best method to select an optimal element combination for the discrimination of site fingerprints, the maximal prediction accuracy (i.e. the percentage of correct assignment of the fish to their capture habitat) was tested according to the methods used (RF, LDA, and ANN) and for each possible

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combination of 1 to N chemical elements ($2 \times \exp^{(N - 1)}$ possible combinations). Furthermore, a cross-validation was performed for each element combination by testing 1000 replicates. For each cross-validation procedure, 75% of the fish (training data set) were randomly chosen to train the classifiers, the remaining 25% being used to measure the quality of prediction. Five hundred trees were built for the random forest method. For more details, see the R script named R_otolith_microchem_elements_and_method_selection.R (Mercier et al., 2011). To complete this analysis, a multivariate analysis of variance (MANOVA) type III (for un-balanced data, 'car' R package) was used between the fingerprints of the three sites.

Lastly, to analyse site fidelity according to sites and life stages, microchemical fingerprints of each macrostructure (see Figure 2, L, S0b, S0m, S0e, W0, S1, W1, etc.) were compared with each other and to the capture fingerprint (C, site signature) with MANOVA type III. All MANOVA were used with the Pillai's trace since it is relatively robust to deviations from multivariate normality (Johnson and Field, 1993). Indeed, half of the elements (i.e. Ba/Ca, Sr/Ca, and Fe/Ca) in the data based on different life stages of Lancieux fishes did not meet normality even after $\log_{10}$ transformation.

All the element ratios were transformed by log10. The threshold for rejection of the null hypothesis was defined at $p = 0.05$ and coded as follow: *: $0.05 > p > 0.01$; **: $0.01 > p > 0.001$; and highly significant ***: $p < 0.001$. All statistical analyses were performed using R software (R-2.15.1 R Development Core Team 2012).

3. Results

3.1. Growth estimation: validation from cohorts and otolith microstructures

Four species were caught at Lancieux Bay: *Hyperoplus lancelolatus*, *H. immaculatus*, *Gymnammodytes semisquamatus* and *Ammodytes tobianus,* the last accounted for 73.04% (n = 642) of the catches. At Lancieux Bay, fish sizes ranged from 38 to 175 mm (Figure 3). A maximum age of 5 years was observed, but most fishes were one or two years old. Irrespective of the sampling period, individuals from the G2 (131.62 ± 7.53 mm) and G3 (146.47 ± 8.34 mm) age classes were detected. At the beginning of the survey, G1 individuals born in spring 2011 (96.71 ± 7.83 mm) were still very abundant in February and April 2012, with an estimated age of around one year old (i.e. G1, Figure 3, black stars). During the survey, two newly recruited juvenile cohorts were detected; the first, born in autumn 2011 (G0 A2011, 41.37 ± 2.37 mm, Figure 3), appeared at the beginning of the survey in February 2012 and grew 7.93 mm between February and March, with a maximum growth rate in May (20.98 mm/month). For this cohort, the growth started to decrease at the end of the summer, was very low during the winter (0.46 mm between November and December) and reached one year of age during the autumn (Figure 3, black stars). Conversely, individuals born during the spring of that year (G0 S2012, 40.64 ± 3.56 mm, Figure 3), recruited in May, had higher growth at the beginning of their life (7.93 mm and 17.98 mm between the first two months, G0 A2011 and G0 S2012, respectively)

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and a higher maximal growth rate in June (34.38 mm/month) than individuals from G0 A2011. Interestingly, at the end of their first year of growth, juveniles were almost the same size regardless of their recruitment period.

Finally, at one year old, individuals of G1 and G0A2011, from whom it was only possible to estimate the season of birth, measured 109.89 ± 4.08 and 120.75 ± 4.50 mm, respectively (Figure 3, black stars).

Fork length (FL) and the otolith total length (Toto) were highly correlated and can be expressed as FL $= 0.0454 \times$ Toto $+ 11.083$ ($R^2 = 0.94$, Df = 41, $p < 2.2e^{-16}$ ***, Figure 4). The mean length of sandeels at one year of age was back-calculated from adult otolith diameter at the end of the first opaque zone of the otolith, and was estimated at 118.33 ± 7.15 mm.

The larval zone had an average diameter of 265.62 ± 21.05 µm (n = 24) and did not change among cohorts (Df = 3, p = 0.30, Figure 5). However, the following first growth zone (i.e. S0) was significantly wider for individuals born in autumn (i.e. G0 A2011, 1927.52 ± 75.00 µm, n = 3) than individuals born in spring (G1, 1323.63 ± 107.01 µm, n = 5; G0 S2012, 1398.17 ± 68.99 µm, n = 5), but also for older individuals (n = 11) (Df = 3, $p = 2.02e^{-05}$ ***, Figure 5). G2 might also be born in spring considering their small S0 zone.

3.2. Significance of microchemical fingerprints in otoliths

3.2.1. Ontogenic and temporal variation in otolith fingerprints

Mg/Ca varied significantly both according to season and fish age (Df = 3, Deviance explained per factor = 77%, $p = 1.45e^{-06}$***) and exhibited higher ratios in juvenile otoliths and during the summer (Figure 6). The microchemical fingerprints of the other elements ratios were not different (mean $\pm$ sd of all individuals: Ba/Ca $= 2.12e^{-06} \pm 6.24e^{-07}$, Sr/Ca $= 4.40e^{-03} \pm 1.02e^{-07}$, Mn/Ca $= 1.19e^{-05} \pm 6.51e^{-06}$, Zn/Ca $= 8.63e^{-06} \pm 7.38e^{-06}$, Cu/Ca $= 8.12e^{-06} \pm 7.95e^{-06}$, and Fe/Ca $= 2.02e^{-03} \pm 2.40e^{-04}$). Meaning the variation in Mg/Ca ratio in otolith fingerprint is more due to seasonal effects and physiological changes during the fish development than to ontogenic habitat changes (see Discussion). Therefore to avoid biaises, Mg/Ca was removed in further analysis.

3.2.2. Discrimination of sites and optimal element combination

The three classification methods performed (LDA, RF, and ANN) provided good maximal accuracy of prediction, falling between 78.44% and 83.79% (Table 1). LDA had the best maximal prediction accuracy (83.79%), and the best element combination was composed of Cu/Ca and Mn/Ca (Table 1). Capture signature in otoliths from Lancieux was significantly different from those of Chausey (Manova, p = 0.0070**) and Rotheneuf (Manova, p = 0.032**) but Chausey and Rotheneuf were not globally different (Manova, p = 0.27). Mn/Ca ratios were significantly higher in otoliths from

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Rotheneuf and Chausey than those from Lancieux (Figure 7). Cu/Ca and Zn/Ca ratios were significantly higher in otoliths from Rotheneuf than the two other sites (Figure 7).

3.3. Comparison of life stage signatures from Lancieux sandeels with signatures of sites of capture (Chausey, Lancieux, and Rotheneuf)

The large majority of the microchemical fingerprints found for the different life stages (i.e. macrostructural zones of otoliths) of Small Sandeels captured at Lancieux always appeared different from the Chausey and Rotheneuf capture signatures (Table 2). Among the elements, Cu/Ca, Mn/Ca, and Zn/Ca ratios were always significantly different from the Rotheneuf capture signature, and Mn/Ca and Fe/Ca ratios differed significantly and the most frequently from Chausey capture fingerprint (Table 2).

The microchemical signatures varied significantly from the centre (larval stage) to the margin of the otoliths. Two main differences occurred between the larval stage (L) and the beginning of the first growth stage (S0b) (Table 3). The larval stage was more enriched in Sr/Ca, Ba/Ca, Zn/Ca, and Cu/Ca than the S0b stage (Table 3). The second significant change was between this latter zone (S0b) and the first mid-growth stage (S0m) (Table 3). Then, the first mid-growth stage (S0m) did not differ significantly to the end of this zone (S0e). These three first zones (L, S0b, S0m) were significantly different from the Lancieux capture fingerprint, unlike for all of the following zones of the otolith (S0e, W0, S1, W1 and C), which did not vary significantly (Table 3).

4. Discussion

4.1. Methodological validations

Results of growth estimation validate the correlation between otolith and fish length and permit to further link the different life stages of otolith to the continuous laser ablation. The definitive length of fish, almost reached at one year of age, was estimated from otoliths at 118.33 ± 7.15 mm and was corroborated by the size estimated from the two cohorts (respectively 109.89 ± 4.08 and 120.75 ± 4.50 mm, see Figure 3, black stars). This is in accordance with Reay (1973), who found fish sizes at one year of age ranging between 110 and 114 mm for two different years. Furthermore, the length of the first growth zone in otoliths can help to detect the season of birth; a small first opaque zone in the otolith corresponds to fish recruited in late spring or autumn and born in early spring, while a large zone corresponds to fish recruited in spring and born in the previous autumn, as Reay (1973) reported. This marked macrostructure of the otoliths was useful to reliably detect ontogenetic stages in the sandeel otoliths, and, given the relatively large size of the otoliths and large identifiable zones in the first year of life, it was possible to perform broad laser ablations providing enough material to detect fingerprint variations.

However, in order to definitely validate the use of microchemical fingerprints, we had to check their stability according to years, seasons, and age classes, as previously described in various fish species

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(Chittaro et al., 2006; Tanner et al., 2011; Mercier et al., 2012; Tournois et al., 2013). Among all the elements analysed in the Small Sandeel otolith, only Mg/Ca differed according to age class and season, which is consistent with several studies thatfoundmagnesium to be physiologically regulated (Martin and Thorrold, 2005; Tanner et al., 2011; Woodcock et al., 2012).

Three methods were used to verify whether microchemical variations in otoliths were reliable for detecting habitat changes. Although the random forest (RF) method has improved discrimination performances for recent microchemistry data sets (Mercier et al., 2012; Tournois et al., 2013), linear discriminant analysis (LDA) had the highest maximal accuracy in our study. This could be explained by the fact that our dataset of capture sites exhibits multi-normality and homoscedasticity (Mercier et al., 2011). The best element combination was composed of Cu/Ca and Mn/Ca, and these elements are generally influenced by terrigenous inputs and tend to decline with distance from the shore (Kremling, 1985; Shiller, 1997; Laes et al., 2007) and have already been used to discriminate among coastal sites (Tanner et al., 2011). Our results suggest that the distinction between the three sites of capture was mainly due to a significant enrichment in manganese (Mn/Ca) in the outer perimeter ofotoliths from Chausey and Rotheneuf. In addition, enrichment in copper and zinc in the coastal sites at Lancieux and Rotheneuf enabled us to distinguish between the three sites. The observed differences could be explained by the geological composition of the sediments and of the water. Despite the low number of individuals in Chausey and Rotheneuf sites, our results highlight the potential capacity of the otolith microchemistry of sandeels to imprint local trace elements and therefore to distinguish marine habitats, even over low geographical gradients, and confirm the interest of multi-elementary signatures as a spatial tracer. However, meaningful ecological interpretations of element concentrations remain difficult to make, especially because more information is required to understand the incorporation processes of chemical elements in fish and sandeel otoliths.

4.2. Site fidelity of the Small Sandeel according to age: insights from microchemical fingerprints of otoliths

Firstly, the microchemical signatures of the Chausey and Rotheneuf sites were never similar to those of Lancieux fishes (40 and 20 km away, respectively) suggesting that: (i) environmental imprints may occur among nearby marine areas, and (ii) there was *a priori* no population connectivity between these intertidal sites. This is in accordance with Jensen et al. (2011), who found high fidelity of *A. marinus* to their night-time burrowing sites, with a range that did not exceed 5km. Nevertheless, it appeared that diurnal movements could extend about 15 km away from night-time burrowing sites (Engelhard et al., 2008 for *A. marinus*). Finally, the swimming capacity of the Small Sandeel (1 to 1.5 km/h) (Kühlmannand Karst, 1967), suggested that fish caught in Lancieux were unable to reach either the Chausey archipelago or Rotheneuf Beach through daily movement, which does not exclude potential migrations over several days. Therefore, as telemetry is difficult for small fishes and traditional

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surveys and acoustic methods do not permit tracking individual behaviour, otolith microchemistry appears to be a potentially useful tool to determine the connectivity between sandeel stocks.

Secondly, the signatures of the larval stage (L) and the early juvenile growth stage (i.e. S0b, the beginning of the juvenile zone), including the metamorphosis stage (Wright, 1993), of fish caught in Lancieux, presented significantly distinct microchemical fingerprints from older stages. This first fingerprint change over the Small Sandeel lifespan could be explained by a change inhabitat, probably when the metamorphosis occurred. Indeed, the size estimated from otoliths, at the beginning of the juvenile zone (S0b), just after the larval stage, ranged between 24.96 ± 0.96 mm and 34.04 ± 0.96 mm. Even if no data were found for Small Sandeel, Wright described *A. marinus* larvae as undergoing metamorphosis over the length range 35 to 55 mm TL, leading to a change from a pelagic to a semi-demersal habitat (Wright, 1993). This early life stage seems to occur for Small Sandeel in coastal waters according to Langham (1971), who never found larvae and post-larvae of the species in the Scottish offshore waters. Our sampling tended to confirm this result, since the smallest size of Small Sandeel settled (metamorphosed) detected at Lancieux was 38 mm. Contrary to the following stages, larvae are notably not in contact with sand during the night, which could explain the change in the microchemical fingerprint. Finally, the change in behaviour and habitat (pelagic to semi-demersal) and the influence of particular ecophysiological characteristics (growth and feeding) of these early stages (larval and during metamorphosis) on the microchemical composition of the otoliths cannot be excluded (Otake et al., 1997; Arai et al., 2000; Chittaro et al., 2006; Tanner et al., 2011).

Interestingly, the signature of the middle of the juvenile zone (S0m) differed from the capture signature of the Lancieux site (C), but not from the end of the juvenile zone (S0e) or from the second winter period (W1). At this stage (S0m), the estimated length of fish ranged from 45.10 ± 5.42 to 54.18 ± 5.42 mm. If differences in the first juvenile phase (S0b) and larval signatures are hypothetically due to a change in ecophase, as previously stated, this could be the beginning of settlement in the site in accordance with the mean sizes of recruitment in our samples of 41.37 ± 2.37 and 40.64 ± 3.56 mm according to the period (February 2012 (G0 A2011) and May 2012 (G0 S2012)). As the end of the first juvenile stage (S0e) did not differ from the next zones and the fingerprint of capture, it could correspond to a real settlement of juveniles. Observed differences in microchemical signatures and the recruitment size could result from: i) a delay of signature incorporation in the otoliths (Yokouchi et al., 2011), or ii) a behavioural difference in habitat use compared to older individuals. Then, the growth and survival of *Ammodytes marinus* larvae are controlled and supported by the zooplankton peak concentration and the increase of temperature enhancing the growth at the optimal time of match-mismatch (Gurkan et al., 2013), so variation in the time of hatching can lead to potentially different larval growth (Wright and Bailey, 1996) and juvenile size at recruitment. Accordingly, our individuals used for microchemistry and the otolith-length relationship were born in different years and probably seasons (G2, G1 (born in spring 2011) and G0 S2012), which might have

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effects on size at arrival at Lancieux compared to those of recruits of the year (G0 A2011 and G0 S2012).

Microchemical results finally support that the Small Sandeel exhibit high fidelity to the Lancieux site, where they have been captured during all stages of their lifecycle after the beginning of their settlement. Based on microchemistry and the sizes of recruitment and juvenile growth, intertidal beaches seem to act as nursery, growth, and resting habitats for the species, especially when they are overwintering (Wright et al., 2000; van Deurs and Steffensen, 2011) and we cannot exclude their potential role as spawning habitat for this species.

4.3. Conclusions

Despite the small size of the sample used for this study, it confirms that exchanges could be limited between sandbanks and sandeel body morphology restricts their movements in the short-term (i.e. foraging behaviour) and longer periods in their lifecycle (Engelhard et al., 2008; Jensen et al., 2011). This method is also able to detect ontogenic habitat shifts in the Small sandeel, notably the settlement stage in intertidal beaches. Otolith microchemistry appears to be a relevant tool for investigating the life history of short-lived fishes such as Ammodytidae. It also provides a complementary approach to molecular methodologies to unravel population mixing on a geographical scale relevant to conservation and management.

Acknowledgements

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1   Table 1: Maximal classification success (± standard deviation, sd) and best combination of

2   elements obtained by three classification methods: linear discriminant analysis (LDA), random

3   forest (RF), and artificial neural networks (ANN). Microchemical fingerprints are inferred from

4   the external zone (signature of capture) of the Small sandeel otoliths from Lancieux (n = 24),

5   Chausey (n = 5), and Rotheneuf (n = 5).

Method	Maximal accuracy (% ± sd)	Combination of elements
LDA	83.79 ± 12.35%	Cu/Ca, Mn/Ca,
RF	79.19 ± 12.67%	Cu/Ca, Mn/Ca, Sr/Ca, Zn/Ca
ANN	78.44 ± 13.79%	Cu/Ca, Fe/Ca, Mn/Ca, Zn/Ca

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1   Table 2: Multivariate analysis of variance (MANOVA **type III**) comparing the different microchemical signatures (corresponding

2   to different life stages) of small sandeel otoliths from Lancieux (n = 24) with capture signatures of the otoliths from Chausey (**n = 5)**

3   **and Rotheneuf (n = 5).** The macrostructure of the Lancieux otoliths is divided into several zones (see materials and methods): L =

4   larvae; S0b = beginning, S0m = middle, S0e = end of first opaque zone corresponding to the first summertime growth period; W0 =

5   first translucent zone corresponding to the first winter growth period; S1 = second opaque zone (second summertime growth period);

6   W1 = second translucent zone (second winter growth period). Indicated p values are the mean of different p values calculated for each

7   element ratio between pairwise analyses (Example: **larval zone of Lancieux (L) versus capture signature of Chausey**). Significant

8   elements are identified for each pairwise analysis (*: $0.05 > p > 0.01$; **: $0.01 > p > 0.001$; ***: $p < 0.001$; and NS = non-significant).

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	Capture signature of Chausey		**Capture signature of Rotheneuf**	
	P value	**Significant elements**	**P value**	**Significant elements**
L	$6.17e^{-06}$ ***	Mn/Ca ***, Sr/Ca ***, Cu/Ca **, Ba/Ca **, Fe/Ca **	$1.85e^{-06}$ ***	Mn/Ca ***, Sr/Ca ***, Cu/Ca **
S0b	$8.38e^{-04}$ ***	Mn/Ca ***, Fe/Ca **, Cu/Ca *	$2.55e^{-04}$ ***	Mn/Ca ***, Cu/Ca ***, Zn/Ca **
S0	$3.89e^{-03}$ ***	Fe/Ca **, Mn/Ca *	$1.03e^{-05}$ ***	Cu/Ca ***, Zn/Ca ***, Mn/Ca **
S0e	$1.54e^{-02}$ *	Mn/Ca **, Fe/Ca *	$1.32e^{-04}$ ***	Cu/Ca ***, Zn/Ca ***, Mn/Ca **

W0	**0.017 ***	Mn/Ca ***	**1.89e^{-04} *****	Mn/Ca ***, Cu/Ca ***, Zn/Ca **, Sr/Ca **, Ba/Ca *
S1	**0.020 ***	Mn/Ca **, Zn/Ca *	**9.34e^{-04} ****	Mn/Ca ***, Zn/Ca ***, Cu/Ca **

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1   Table 3: Multivariate analysis of variance (MANOVA) comparing, from the Lancieux Small Sandeels otoliths (n = 24), the

2   microchemical fingerprints during the ontogeny.. The otoliths were divided into several ontogenic stages inferred from macrostructural

3   patterns (see materials and methods): L = larvae; S0b = beginning, S0m = middle, S0e = end of first opaque zone corresponding to the

4   first growth period; W0 = first translucent zone corresponding to the first growth stop; S1 = second opaque zone (second growth

5   period); W1 = second translucent zone (second growth stop period). The microchemical fingerprints of each of these ontogenic stages

6   were compared to each other. Indicated p values are a mean of different p values calculated for each element ratio between pairwise

7   analyses (Example: **larval zone of Lancieux (L) versus capture signature of Lancieux).** Significant elements are identified for each

8   pairwise analysis (*: $0.05 > p > 0.01$; **: $0.01 > p > 0.001$; ***: $p < 0.001$; and NS = non-significant).

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		S0b	S0m	S0e	W0	S1	W1	Capture signature Lancieux
L		**1,13 e^{-09} ***** Sr/Ca***, Ba/Ca***, Zn/Ca**,	**1.14e^{-06} ***** Sr/Ca***, Cu/Ca***, Zn/Ca***, Ba***,	**3.12e^{-05} ***** Sr/Ca***, Zn/Ca***, Ba/Ca***, Cu/Ca**,	**1,57e^{-05} ***** Ba/Ca***, Sr/Ca**, Cu/Ca**,	**4,53e^{-04} ***** Sr/Ca***, Ba/Ca***, Zn/Ca***,	**8,02e^{-03} **** Zn/Ca***, Cu/Ca*	**2,92e^{-03} **** Ba/Ca***, Sr/Ca *

	Cu/Ca*	Mn/Ca*	Mn/Ca*	Zn/Ca**	Cu/Ca*		
S0b		$7,16e^{-03}$ ** Sr/Ca**, Cu/Ca*	$3,43e^{-03}$ ** Sr/Ca***	$4,85e^{-5}$ *** Sr/Ca***	$1,49e^{-02}$ * Sr/Ca**	$3,82e^{-02}$* Sr/Ca**, Zn/Ca*	$1,07e^{-02}$* Sr/Ca ***
S0m			NS	$3,02e^{-04}$** Sr/Ca***	$3,66e^{-02}$* Fe/Ca*	NS	$1,28e^{-02}$ ** Cu/Ca *, Zn/Ca *
S0e				NS	NS	NS	NS
W0					NS	NS	NS
S1						NS	NS

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1   Figure 1: Location of the three intertidal sampling sites of Small Sandeels in the Norman-

2   Breton Gulf (south-western English Channel).

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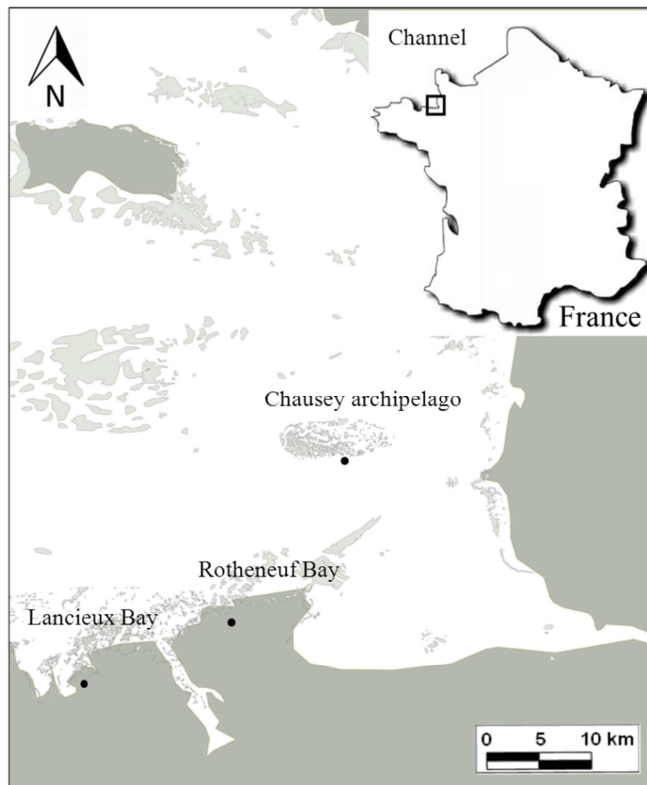
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1   Figure 2: Microscopic photographs of an otolith from *Ammodytes tobianus* after sagittal

2   section (transmitted light). (A): Focus on the circular larval zone: (1) nucleus, (2) daily

3   increments, (3) end of the circular larval zone. (B): Otolith after the linear continuous ablation

4   (X): (4) circular larval zone = L, (5) first summer growth season (S0) divided in three zones:

5   beginning = S0b, middle = S0m, end = S0e, (6) first winter growth season = W0, (7) second

6   summer growth season = S1, (8) second winter growth season = W1, (9) edge of otolith and

7   signature of the capture site = C. Black horizontal arrows represent the distance considered to

8   calculate the means of element ratios in different identified zones of the otolith.

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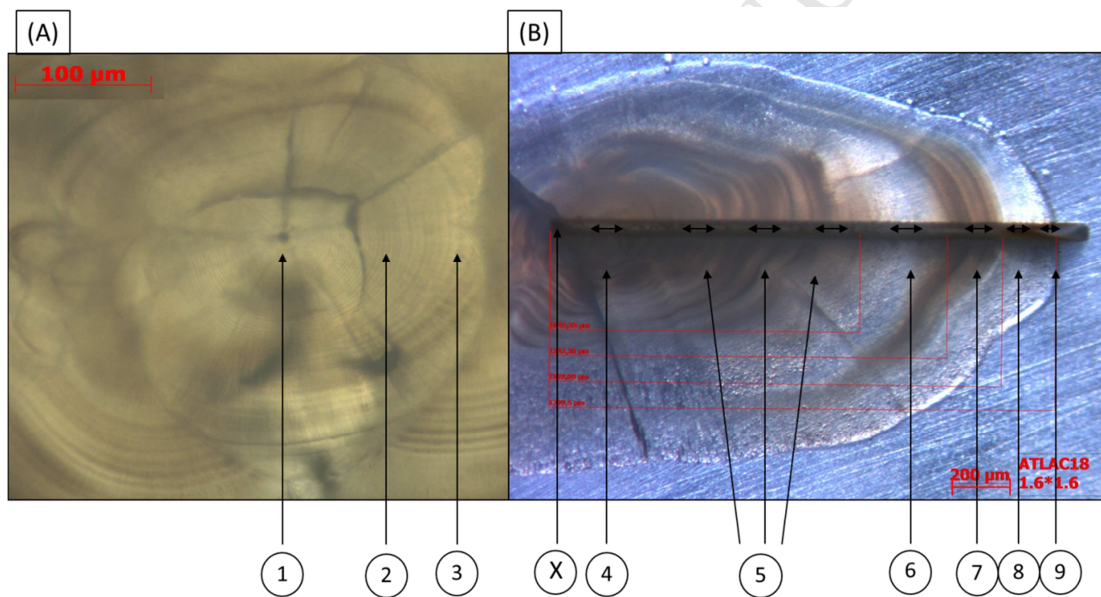
1   Figure 3: Mean (solid lines) and individual sizes (mm) (points) distribution of Small sandeels

2   sampled from February 2012 to January 2013 (with confidence interval of 95% around the mean

3   in grey). Codes represent identified cohorts (i.e. individual of the same age class: G0, G1, etc.),

4   back-calculated season of birth (S: spring; A: autumn), and year of birth. G0 A2011 = group 0

5   born in autumn 2011, G0 S2012 = group 0 born in spring 2012, G1 = group 1 born in spring

6   2011, G2 = group 2, G3 = group 3, G5 = group 5. Accurate estimation of size reach by fish at one

7   year of age was only possible for two cohorts (**G1** and **G0 A2011**), and represented by black

8   stars.

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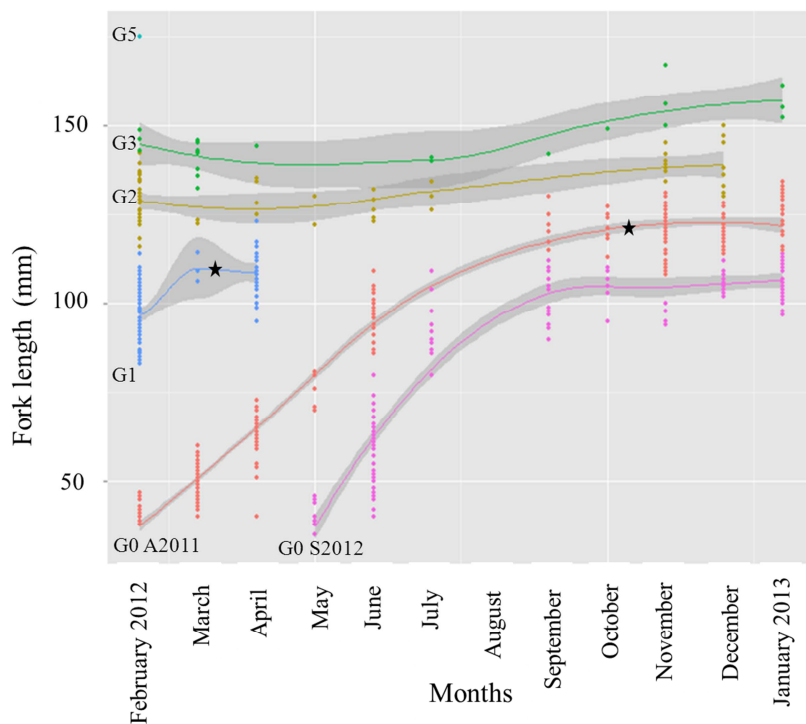
1   Figure 4: Relationship between fish fork length (FL in mm) and total length of sagittal otolith

2   (mm) (antero-posterior axis, n = 44).

3
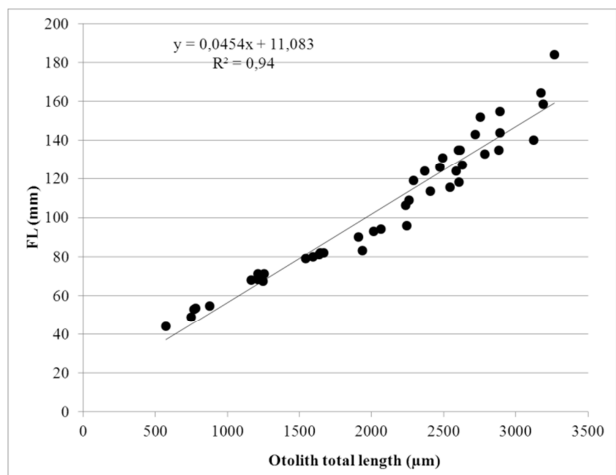
4

5

1 Figure 5: Comparison between otolith lengths at the larval stage (L) and at the first summer

2 growth (S0) according to different cohorts captured at Lancieux (n = 24). Cohorts used are G1 =

3 group 1 born in spring 2011, G0 A2011 = group 0 born in autumn 2011, G0 S2012 = group 0

4 born in spring 2012, G2 = group 2 years old. Letters in superscript indicate significant differences

5 ($p < 0.05$) between the cohorts (S0) according to a linear model. No significance difference

6 occurs at larval stage (NS).
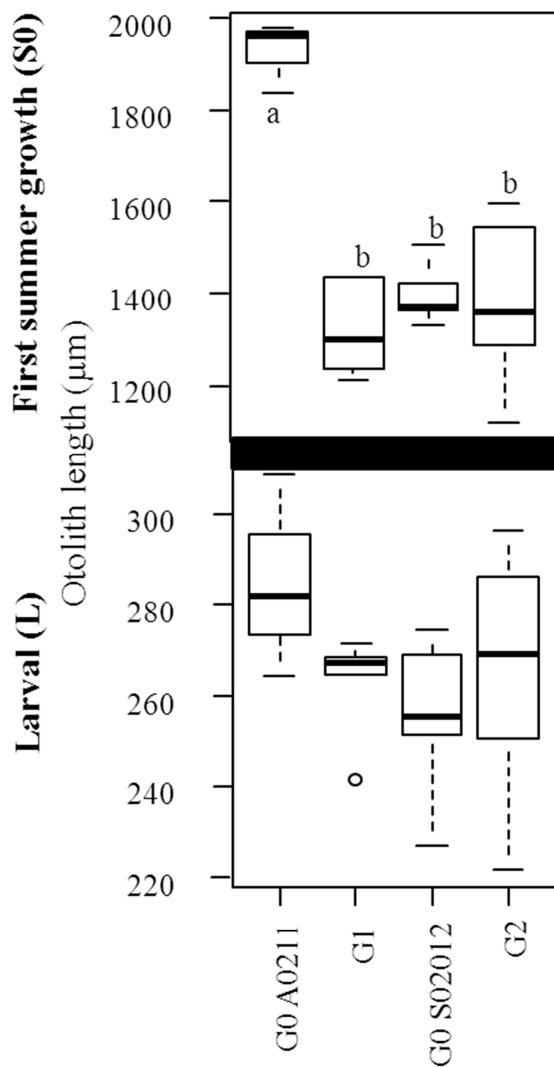
7

8

1 Figure 6: Comparison of Mg/Ca ratios from juveniles and adults otoliths according to months of

2 capture at Lancieux (n = 24; adults in July (Ajul, n=5) and November (Anov, n=9), juveniles in

3 February (Jfeb, n=5) and July (Jjul, n=5)). Letters in superscript indicate significant differences

4 ($p < 0.05$) from a linear model.
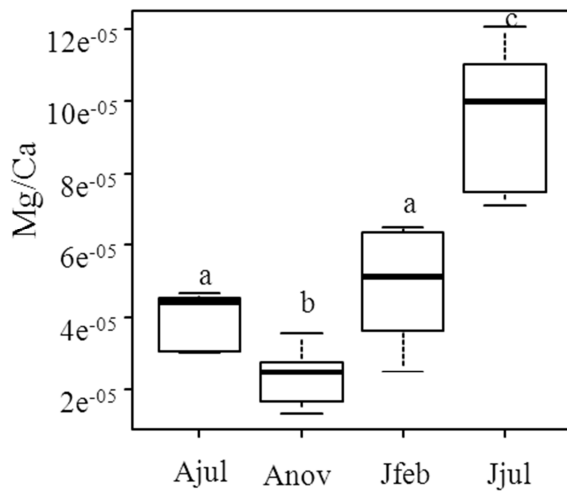
5

1   Figure 7: Boxplots comparing different element ratios from Small sandeels otoliths from the three

2   sites of capture. Element ratios are inferred from the external zone of the otoliths (capture

3   fingerprint) of Lancieux (n = 24), Chausey (n = 5), and Rotheneuf (n = 5). Letters in superscript

4   indicate significant differences (MANOVA, p < 0.05) between sites, when present.

5

6

7
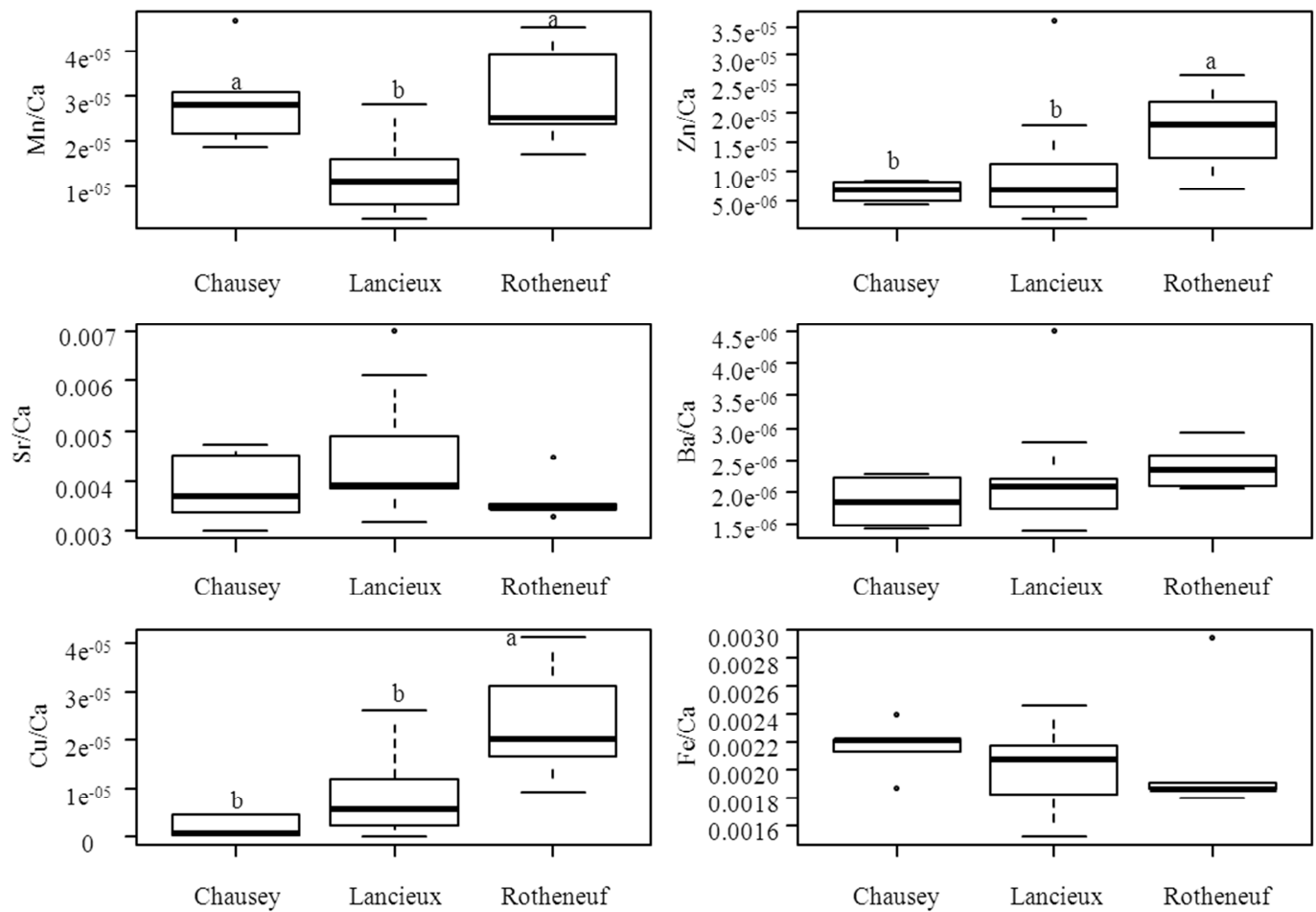
Highlights

- Otolith microchemical fingerprints are studied for the first time in Ammoditydae.

- Small sandeels otoliths have significant distinct microchemical signatures at reduce spatial scales (from 20 to 40 km).

- **Ontogenic habitat shift is detected by the microchemical signatures.**

- Microchemical signatures revealed high site fidelity and low dispersion after the settlement.

- Microchemical fingerprints appear relevant to unravel population mixing and habitat use for small temperate fish species.