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Dissertação no âmbito do Mestrado em Ecologia orientada pelo Doutor Filipe Miguel Duarte Martinho e pelo Professor Doutor Miguel Ângelo do Carmo Pardal e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra.

Setembro de 2018

Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da

Universidade de Coimbra

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Resumo

A variação da composição elementar dos otólitos em relação à composição elementar na água é uma ferramenta muito útil para determinar os movimentos dos peixes, as suas rotas de migração e também os padrões de uso do habitat. Neste estudo, foi realizada uma experiência laboratorial para avaliar os efeitos de variações na salinidade (5, 18, 30) e temperatura (16ºC, 21ºC) na composição elementar de otólitos de juvenis de *Solea senegalensis*, uma espécie com grande importância económica para as pescas e também para aquacultura. Esta espécie migra entre as águas marinhas e salobras, especialmente durante o primeiro ano de vida. Os resultados deste trabalho mostram a habilidade de Sr:Ca e Ba:Ca em distinguir salinidades altas e baixas, mas não em salinidades intermédias características de ambientes tipicamente estuarinos, com água salobra. Não houve efeito significativo da temperatura nos elementos referidos anteriormente, enquanto para Mn:Ca, ^{24}Mg:Ca e ^{25}Mg:Ca não houve diferenças entre os tratamentos. Quer a temperatura quer a salinidade afetaram significativamente o coeficiente de partição do Sr (D_{Sr}) , enquanto que para o D_{Ba}, foi também detetada uma interação entre estes fatores.. D_{Mn}, D_{24Mg} e D_{25Mg} também apresentaram comportamento diferente: D_{Mn} apresentou diferenças em salinidade e na interação entre salinidade e temperatura, enquanto D_{24Mg} apresentou diferenças em salinidade e temperatura e D_{25Mg} apenas diferenças em salinidade. Os regimes de temperatura e salinidade utilizados no presente estudo abrangem aqueles ocupados por juvenis no seu habitat natural, e, portanto, fornecem um teste realista para o uso da microquímica de otólitos para compreender a história ambiental de indivíduos desta espécie.

A composição elementar do otólito resulta de vários fatores ambientais, apoiando a suposição de que variações ambientais são refletidas na composição do otólito. Os resultados deste trabalho indicam a necessidade de combinação de experiências de laboratório com estudos de campo para melhor compreender os processos envolvidos na incorporação de elementos na matriz do otólito e também para interpretar padrões específicos para a espécie na química de otólitos.

Palavras chave: Otólitos; Microquímica; *Solea senegalensis*; Temperatura; Salinidade

Abstract

Variation of the elemental composition of otoliths relative to the elemental composition in water is a very useful tool to determine fish movements, their migration routes and patterns of habitat use. In this study, we performed a laboratory experiment to evaluate the effects of variations in salinity (5, 18, 30) and temperature (16ºC, 21ºC) in the otolith elemental composition of juvenile *Solea senegalensis*, a species of high economic importance for both fisheries and aquaculture, which migrates between marine and brackish waters, especially during the first year of life. Our results show the ability of both Sr:Ca and Ba:Ca to discriminate between high and low salinities, but not at the intermediate levels representing typical estuarine brackish environments. There was not a significant effect of temperature for the previous elements, whereas for Mn:Ca, ^{24}Mg:Ca and ^{25}Mg:Ca there were no differences between salinity, temperature and interaction between salinity and temperature. The temperature was at a high level from Sr (D_{Sr}), while that at D_{Ba}, an interaction between these factors was also detected.. D_{Mn}, $D^{24}{}_{Mg}$ and $D^{25}{}_{Mg}$ also had different behavior: D_{Mn} presented differences in salinity and in the interaction between salinity and temperature, while $D^{24}{}_{Mg}$ presented differences in salinity and temperature, and $D^{25}{}_{Mg}$ had only differences in salinity. The temperature and salinity regimes used in the present study cover those occupied by juveniles in their natural habitat, and therefore provide a realistic test for the use of otolith microchemistry to understand the environmental history of individuals of this species.

The elemental composition of the otolith results from several environmental factors, supporting the assumption that variations in the environment are reflected in the composition of the otolith. The results of this work indicate the need for the combination of laboratory experiments with field studies to better understand the processes involved in incorporating elements into the otolith matrix and to interpret species-specific patterns in the chemistry of otoliths.

Keywords: Otoliths; Microchemistry; *Solea senegalensis*; Temperature; Salinity

Chapter 1 - Introduction

1.1 Estuaries as nursery areas for marine fishes

Estuaries are biosystems composed of several different habitats, with physical, chemical and biological links between them (Pihl et al., 2002). These areas are important nursery sites for marine fish, such as flatfishes, whose shallow waters provide refuge from predators and abundant food resources (Beck et al., 2001; Bento et al., 2016; Cabral et al., 1999; Dahlgren et al., 2006; Freitas et al., 2012; Sheaves et al., 2006; Vasconcelos et al., 2008; Vasconcelos et al.,2012). In these areas, several ecological processes support higher contributions to the adult stocks through the combination of density, growth, survival and movement to habitats used by adult individuals (Cabral et al., 2007).

One important aspect to be considered when evaluating the importance of a habitat fragment in an estuary is the proximity and/or degree of connectivity to other habitat areas (Pihl et al., 2002). Connectivity is a central concept in marine ecology as it appertains to the dependence of fish production and population dynamics on dispersal and migration among multiple habitats (Cowen et al., 2000; Gillanders et al., 2003; Pihl et al., 2002; Vinagre et al., 2008). Connectivity between estuaries and coastal areas is of the utmost importance due to its implications for population dynamics, colonization patterns of new habitats and populations resilience to fisheries (Tanner et al., 2013; Thorrold et al., 2001).

Marine fishes with ontogenic migrations (i.e. throughout their life cycle) share a common life cycle (Grioche et al., 1997; Koutsikopoulos & Lacroix, 1992; Martinho et al., 2013; Primo et al., 2013), in which after hatching, larvae are transported into estuaries or coastal areas due to the influence of tides and vertical movements of water caused by the lunar-tide cycle. Other factors such as speed, direction and intensity of wind and currents, olfactory signals, turbidity and substrate composition may be important in this larval transport (Gibson, 2003; Primo et al., 2012). Already in these areas, they suffer metamorphosis and grow to sub-adult stages, when they migrate to typical adult offshore habitats (Martinho et al., 2013; Primo et al., 2013; Savina et al., 2016; Tanner et al., 2011).

The migratory movements of larvae and juveniles into estuaries is one of the most important stages, as it is acknowledged to define the success of recruitment (van der Veer et al., 2000; Able, 2005; Dahlgren et al., 2006; Pihl et al., 2002; Vasconcelos et al., 2007; Vasconcelos et al., 2008). Defined as "the number of individuals that reach a particular age to join a specific part of the population" (Van der Veer et al., 2000), recruitment varies from year to year and it is generally high because the adult population incorporates members of several age classes. Recruitment is strongly affected at various stages of the life cycle, but it is in the transition from larvae to juveniles that there is a greater effect. Furthermore, other factors, such as fisheries, location within the range of the species, species type, and feeding type may cause recruitment variability (Van Der Veer et al., 2000). Given their importance for fish population dynamics and life cycle, it is therefore essential to study the mechanisms related to the colonization and exploitation of estuarine areas, as well as the degree of connectivity between estuaries and coastal areas, to enable the development of effective management and conservation strategies of both populations and habitats.

1.2 Reconstructing fish ontogenic movements

Some of the best approaches for reconstructing fish movements and migrations over time include direct tracking of individuals using physical tags (Loher & Seitz, 2006; Thorrold et al., 2002) and indirect tracking of individuals or populations through natural tags, such as genetic profiles (e.g. microsatellites, SNIPs) and chemical markers (e.g. otolith microchemistry, stable isotopes) (Campana, 1999; Lü et al., 2014; Purcell et al., 2006).

Physical tags allow an easy study of movements and migrations, population number estimates, the analyses of fish growth and predictions of fish mortality. Furthermore, some types of tracking are a cost-effective procedure that can be applied to fish of several sizes and species, can be used in large numbers and it is easily detectable. Some disadvantages in using physical marks are the difficulty in tagging and recapturing (Thorrold et al., 2002), and the lack of information on the time between release and recapture. Physical tags can also affect growth, health and survival of the individuals because it involves some invasive procedure, such as the piercing of the skin (Neves et al., 2018), as well as the behaviour and swimming performance (Bolle et al., 2005; Loher & Seitz, 2006; Neves et al., 2018; Thorstad et al., 2013)

Chemical markers can be obtained from otoliths, scales, fin rays and bony structures. They are of fast application, low cost and high retention (Lü et al., 2014), can be applied at all stages of the life cycle, from eggs to adults (Campana, 1999), some sampling are non-destructive as, for example, spicule analysis (Purcell et al., 2006) but some others are destructive such as otoliths analysis. Chemical markers have the advantage that all individuals in a population are marked. Opposing to studies of species recruitment and migration, chemical tags are not applicable to other fields such as fish behaviour and physiology. Within chemical markers, the microchemical composition of otoliths has been widely used in studies of recruitment, connectivity patterns and migrations of different species (Elsdon et al., 2008; Elsdon et al., 2004; Gillanders et al., 2003; Reis-Santos et al., 2012; Reis-Santos et al., 2013).

Otoliths are rigid calcium carbonate structures located directly behind the brain of teleost (bony) fish (Campana, 1985), which are directly involved in the detection of sound and related to balance and orientation (Campana, 1999). Bony fish have three pairs of otoliths, sagittae, lapilli e asterisci (Campana, 1999; Campana, 1985), out of which the most commonly used in fish ecological studies are the sagittae. Otolith microstructure and microchemistry have vast applications in the research of fish biology, namely to determine the sites of origin, migration patterns, age and growth, as well as the duration of the different life stages of fish (Campana, 1985; Fox et al., 2007; Martinho et al., 2013; Morrongiello et al., 2014; Reis-Santos et al., 2013).

The use of otoliths as natural tags is possible because otoliths are metabolically inert (Campana, 1999; Gillanders et al., 2003; Tanner et al., 2013; Vasconcelos et al., 2007; 2008) and are not resorbed over time, grow continuously (form daily and annual growth patterns) (Reis-Santos et al., 2008; 2013; Tanner et al., 2012) and incorporate trace elements as they develop (Cuveliers et al., 2010; Poulain et al., 2015; Reis-Santos et al., 2012; Vasconcelos et al., 2007; 2008). Analysis of element composition of different otolith strata has been used as a tool for identifying individuals that have different migratory patterns, disclosing populations or fish stocks structure, the birth origin of distinct populations, and identifying estuarine habitats and habitat connectivity patterns (Elsdon et al., 2008; Gillanders, 2002; Reis-Santos et al., 2013; Thorrold et al., 2001; Vasconcelos et al., 2007)

Before reaching the otoliths, most dissolved trace elements have to cross several biological barriers, such as the gills or the intestinal wall, and then through the bloodstream and into the endolymph, where they are precipitated into the otolith (Campana 1999). The elemental composition of otoliths is generally influenced by the concentration of the elements in the surrounding water, providing a natural tag in which all individuals in same environment are marked similarly (Elsdon et al., 2008; Guelinckx et al., 2008; Izzo et al., 2018; Sturrock et al., 2012; Swearer et al., 2003).

This implies that fish living in different environments within an estuary are frequently characterized by a different otolith chemical composition, representing an elemental fingerprint that can place an individual or a population in a habitat that contains the same elements (Campana, 1999; Reis-Santos et al., 2013; Swearer et al., 2003; Vasconcelos et al., 2008). Otolith elemental composition can be used for reconstructing migration patterns along the sea-estuary-river continuum, through their Strontium (Sr) and Barium (Ba) contents (Campana, 1999; Miller, 2011; Reis-Santos et al., 2013). Sr and Ba water concentrations tend to be related in an inverse manner in fresh and saltwater: freshwater is known to have lower concentration of Sr and higher concentrations of Ba, whereas saltwater is characterized by lower concentrations of Ba and higher concentrations of Sr (He & Xu, 2015; 2016; Reis-Santos et al., 2013; Sturrock et al., 2012). Barium helps discriminating the effect of salinity from the effect of temperature in the incorporation of Strontium. As so, to avoid misleading conclusions about fish migrations, these two elements must be analysed together in order to distinguish the two effects (Campana,1999; Martin & Wuenschel, 2006).

Although the relationship between Sr:Ca and Ba:Ca concentrations in saltwater and freshwater have been regularly used to discriminate habitat use patterns in fish with ontogenic migrations worldwide, there is still some controversy regarding their discriminatory ability in transition areas, such as estuaries (Elsdon et al., 2008; Elsdon & Gillanders, 2004; Martin & Wuenschel, 2006; Reis-Santos et al., 2013). Nonetheless, recent work confirmed the correlation between Sr:Ca and Ba:Ca concentrations in otoliths and in the surrounding water (Izzo et al., 2018), showing that ecological niches can influence the otolith concentration ratio of Sr:Ca, whereas the same temperature niches did not affect Ba incorporation in otoliths. Hence, salinity seems to be the primary environmental driver of otolith Ba uptake (Izzo et al., 2018).

Several factors may contribute to variations in otolith elemental composition, particularly in marine species with ontogenic migrations between the ocean and estuaries, which tend to impair our interpretations of habitat use. There can be a contribution of both external factors, such as water temperature and salinity, ambient element concentrations, and internal factors, such as the genetic background and fish diet (Cuveliers et al., 2010; Gillanders, 2005; Gillanders et al., 2003; Izzo et al., 2018; Reis-Santos et al., 2012). In addition, variations in otolith elemental composition also seem to be species-specific (Cuveliers et al., 2010; Reis-Santos et al., 2012). According to (de Vries et al., 2005), the elemental incorporation of Ba is positively related to the increase of the Sr and Mg concentration, and Sr incorporation was positively related to the concentration of Mg and Mn. Recent studies have underlined that all processes affecting ion transport, binding and availability for incorporation also have the potential to influence otolith chemistry (Izzo et al., 2018). In general, the Ba:Ca ratio is mainly affected by environmental conditions, and mediated by diet and growth rates, whereas other physiological processes are less influent. Sr:Ca ratio is strongly affected by physiological processes, namely reproduction, besides some environmental factors, such as temperature and salinity (Gillanders, 2005; Izzo et al., 2018). Sr:Ca assimilation is also higher in younger fish, suggesting a relationship between uptake and fish age (Grammer et al., 2017).

Temperature and salinity influence the concentration of elements in seawater and do not vary independently of one another, while on the other hand, elemental concentration in seawater can also vary over small spatial scales, both vertically in the water column and horizontally between different water bodies (Elsdon & Gillanders, 2002). For instance, the influence of salinity on the elemental concentration ratios in otoliths generally rises with increasing temperature (Elsdon & Gillanders, 2002). Hence, before linking otolith chemistry to a fish life history or to differential habitat use patterns, it is necessary to evaluate the degree to which they can be determined with exactitude (Reis-Santos et al., 2013; Tanner et al., 2013).

Previous studies have highlighted the importance of interactions between the previous factors when discriminating estuarine habitat use by juvenile fish, as well as the species-specific patterns of element uptake. Hence, determining the effects of extrinsic factors on the incorporation of elements into otoliths of fish with ontogenic migrations will allow increasing the potential to interpret their temperature and salinity histories based on otolith microchemistry (Elsdon & Gillanders, 2002).

1.3 Study species

Solea senegalensis is a flatfish species with high commercial value, especially in adult stages, for both commercial fisheries and aquaculture (Castelo Branco et al., 2006). This species has a wide distribution along the eastern Atlantic (Castelo Branco et al., 2010) up to the Bay of Biscay (Cabral & Costa, 1999) to Senegal (Vinagre, 2007), and in the western Mediterranean (Castelo Branco et al., 2010).

In the Portuguese coast, this species occurs mainly in the central estuaries, where salinity, prey variability, sediment type, depth, optimum temperature are important factors for the maintenance of the species (Vasconcelos et al., 2010). There are several important nursery areas for this species, namely the Tagus and Sado estuaries (Primo et al., 2013), the Ria Formosa (Cabral & Costa, 1999) and the Ria de Aveiro (Primo et al., 2013). Larval stages can also be found in the remaining Portuguese estuaries but in smaller numbers, (Primo et al., 2013), whereas juveniles do not appear in low coastal areas (Tanner et al., 2013). *Solea senegalensis* has the typical life cycle of a marine fish with ontogenic migrations, in which adults spawn offshore, larvae and juveniles migrate to estuaries, where they spend up to two years in estuaries before migrating to shelf waters (Primo et al., 2013; Tanner et al., 2013), and finally, adults return to the ocean to spawn. In the Portuguese coast, sexual maturity is attained at 3 - 4 years old (Vinagre, 2007). Overall, recruitment and spawning patterns show a high inter-annual variability, which may be related with the particular environmental conditions found along the Portuguese coast, such as specific hydrographic circulation and spring/summer upwelling events (Vinagre et al., 2013).

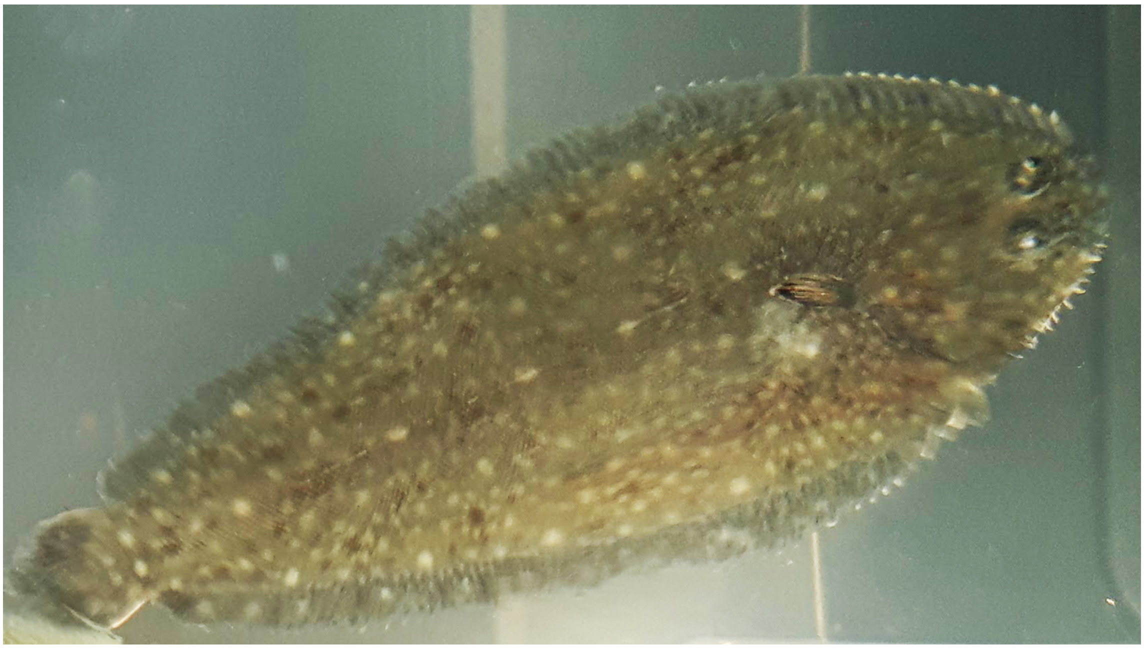
The choice between estuarine nursery areas depends on multiple factors, which are not easily identifiable because estuaries have a complex and highly variable nature (Vinagre et al., 2008). Despite this, some authors consider that this species prefers larger estuaries as nursery grounds (Primo et al., 2013), favouring seagrass beds (Vasconcelos et al., 2010), relatively high temperatures, fine sediment with some percentage of mud, as well as high and stable salinity (Primo et al., 2013; Vinagre et al., 2008; 2009). It is also reported that *S. senegalensis* prefers shallow areas and areas closer to the estuary (Primo et al., 2013; Vinagre et al., 2009). Its habitat use patterns are affected by the lunar cycle, with some migrations occurring between different areas of the same estuary (Vinagre et al., 2006), and also by biotic factors, such as prey availability and predation (Primo et al., 2013; Vinagre et al., 2009).

However, further information is still required on small scale habitat use patterns within estuaries as wells as on the colonization patterns. Research in the area is of the utmost importance and a significant step to understand how abiotic and biotic factors can affect the incorporation rates of elements from water into fish otoliths.

1.4 Objectives

The objective of this work was to evaluate the effects of salinity and water temperature on the chemical composition and uptake (Sr, Ba, Ca, Mn, ^{24}Mg and ^{25}Mg) of juvenile *S. senegalensis* otoliths in a controlled laboratory experiment. Individuals were exposed to multiple environmental variables, including salinity and temperature, representative of the river-estuary-sea gradient, to allow reconstructing different habitat use patterns and migrations.

We hypothesize that otolith chemical composition of juvenile individuals of *S. senegalensis* reflects the salinity, temperature and water element concentrations of their habitat, elucidating different habitat use patterns along the estuarine gradient. We also hypothesize that interactions between different water properties such as temperature and salinity influence element uptake in juvenile sole otoliths.



Chapter 2 - Materials and Methods

2.1 Experimental design

Solea senegalensis juveniles with an average total length (TL) of 9.7cm (± 5.1 cm), approximately 7 months old, were obtained from a hatchery and acclimated to laboratory conditions over 30 days in 200L aquariums with artificial seawater (TMC REEF - Premium REEF Salt), equipped with life support systems (aeration and filtration), at constant temperature (19ºC) and salinity (20). After this period, and to mark the beginning of the experimental procedure, all fish were submitted to a bath of Alizarin Red S (ARS) with a concentration of 300mg/L for 24 hours, following the procedures described in Liu et al. (2009).

After the ARS bath, all fish (n=119) were measured and randomly distributed to 18 20L aquaria at a density of 6 to 7 individuals per aquarium to initiate the acclimation to the test conditions. At this stage, all aquaria, equipped with aeration and filtration systems, had the same temperature and salinity (19ºC and 20, respectively) to minimize the stress induced from transferring fish from the ARS bath. Water temperature and salinity in each aquarium was gradually altered over a 21-day period to acclimatize fish to the defined temperatures (16, 21°C) and salinities (5, 18 and 30) of the experimental treatments. Photoperiod was established as 12h day (dim) and 12h night (dark) daily cycles to better simulate natural conditions. The final experimental design was composed of 6 test conditions (2 temperatures x 3 salinities) with three replicates each (Figure 1). These salinity and temperature ranges were selected because they represent typical environmental conditions *S. senegalensis* juveniles experience over spring and summer during the time they use estuaries as nurseries (Vasconcelos et al., 2010; Vinagre et al., 2008).

During the experiment, salinity was controlled by adding fresh dechlorinated water with artificial marine salt (TMC REEF- Premium REEF Salt) to match each test condition (5, 18 and 30). Water temperatures were controlled through an air conditioning system, which was kept constant at 21ºC and an open water bath system connected to a chiller kept stable at 16ºC. Approximately 40% of the water was changed twice a week (every 2-3 days). Salinity, water temperature, oxygen (mg/L) and pH were measured daily. Ammonia, nitrites and nitrates were analyzed daily during quarantine, acclimatization and the first days of the experiment to ensure the best possible water quality. After this period, these compounds were measured once a week.

Fish were kept in the test aquaria for 98 days between December 20, 2017 and March 28, 2018. During the experiment, fish were fed daily with specialized food pellets until apparent satiation, corresponding to 1-2% of their body weight. Excess food and any fragments were siphoned after one hour. During the experiment, dead fish were removed from the aquarium, TL and wet weight were determined, and then carcasses were discarded (N=7).

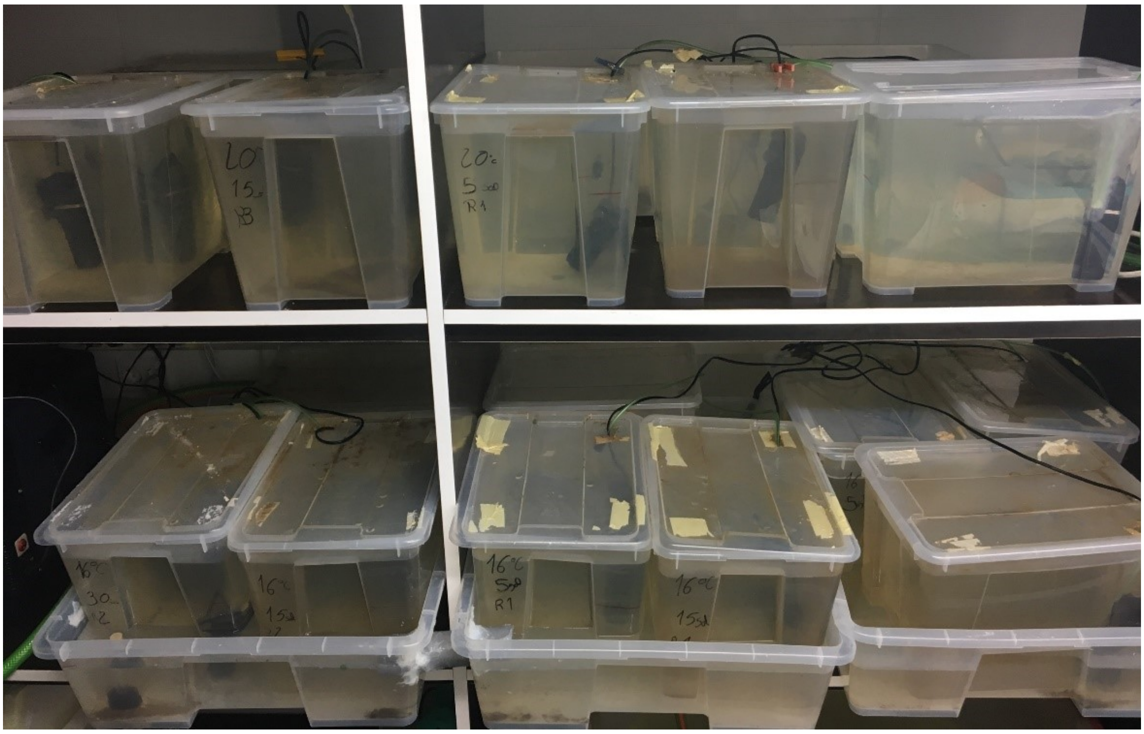


Figure 1- Experimental design showing the test aquarium and the open water bath (bottom row).

2.2 Water analyses

Every two weeks, duplicate water samples from each experimental condition were collected with polypropylene syringes to characterize elemental concentrations. Water samples were filtered (GF/F, 0.2 µm) into 60mL flasks and acidified with concentrated ultrapure HNO_3 (nitric acid) in a 1:50 proportion and stored at 4 °C until analysis.

Prior to elemental quantification by inductively coupled plasma mass spectrometry (ICP-MS), each water sample was acidified in a proportion of 1:10, by adding 3mL of a previously distilled 10% nitric acid solution to 3mL of sample. Water samples were analyzed for ^{43}Ca, ^{88}Sr, ^{138}Ba, Mn, ^{24}Mg and ^{25}Mg by Inductively coupled plasma mass spectrometry (ICP-MS), using a Thermo Fisher Scientific Model iCAP Q spectrophotometer (Bremen, Germany), located at the Physics Department, University of Coimbra, Portugal (Figure 2). The calibration of ICP-MS measurements was done using the 5-point calibration curves per element. Standard solutions were prepared with dilutions of a multielement standard (92091, Periodic table mix 1 for ICP, Sigma-Aldrich), and a standard curve was generated per element at 0, 1, 10, 100, 1000 and 2000 ppm concentrations. Blank controls consisted of ultrapure acidified 10% water. Standards and blanks were read at the beginning of the readings. The detection limits were 1.1213, 0.8544, 2.0350, 0.0122, 0.0188 and 0.0380 ppb for ^{24}Mg, ^{25}Mg, ^{43}Ca, Mn, Sr and Ba, respectively.

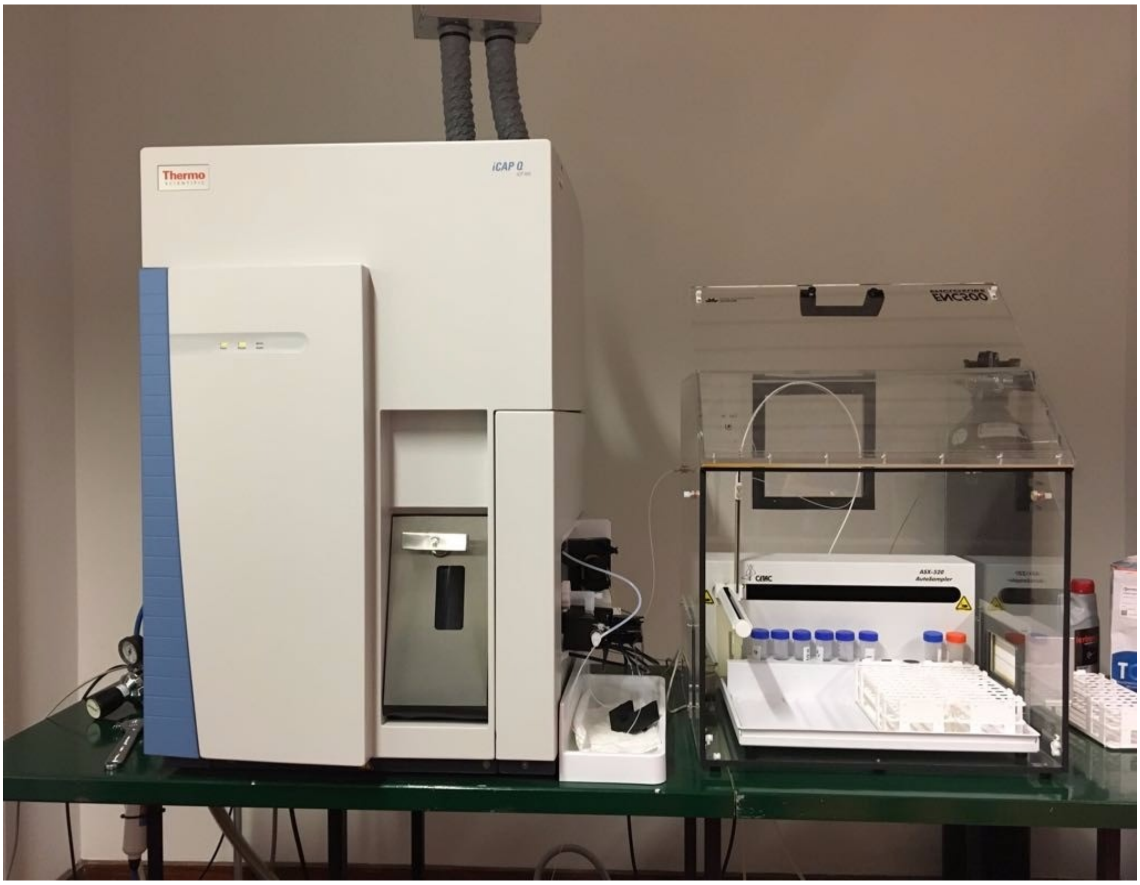


Figure 2 - ICP-MS equipment (Thermo Fisher Scientific Model iCAP Q) at the University of Coimbra.

2.3 Otolith analyses

After the 98-day experimental procedure, all fish were measured and weighed, and their sagittae otoliths removed, cleaned with distilled water and left otoliths were mounted on microscope slides with cyanoacrylate glue. Subsequently, otoliths were polished with 3µm lapping film to expose an even surface between the core and the edge of the otolith, and sonicated until the otolith was released, as in Tanner et al., (2013) double-sided tape was attached to microscope glass slides previously marked with longitudinal lines to guide the placement of the otoliths, with the core slightly offset from the line. A total of 40 otoliths (10 x 4) were fixed in each microscope slide for further analysis.

Otolith chemical analysis followed Reis-Santos et al (2012). A 213nm high performance UV (Nd: YAG) laser microplate coupled to an Agilent 7900cs inductively coupled plasma mass spectrometer (ICP-MS) was used to quantify ^{24}Mg, ^{25}Mg, ^{55}Mn,^{44}Ca, ^{88}Sr, and ^{138}Ba, concentrations in otoliths (Figure 3).

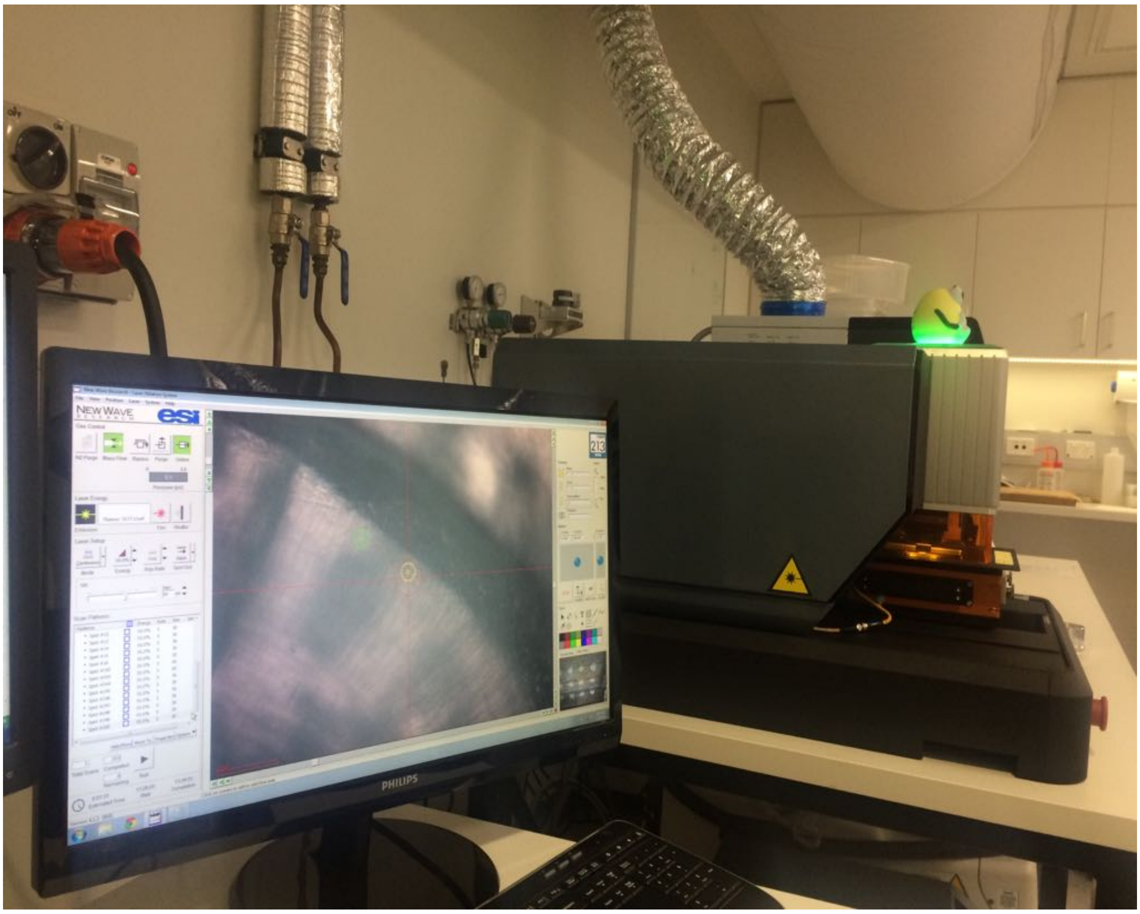


Figure 3 - Laser ablation equipment at the University of Adelaide (Australia).

Ca was used as an internal standard to normalize variations in ablation yield. In all otoliths, three spots (with a 25 µm diameter laser) were ablated in the marginal edge of the otolith (Figure 4) and representing otolith material deposited during the experimental period and recent elemental incorporation. Laser ablations occurred in a sealed chamber with resulting analyte transported to the ICP-MS via a smoothing manifold in an Ar and He stream. A glass certified reference material (NIST 612 -

National Institute of Standards and Technology) was analyzed at the start and end of each session and after every 10 otoliths to correct for mass bias and machine drift. External precision (% relative standard deviation) was determined based on a calcium carbonate certified reference material, MACS-3 (United States Geological Survey) (RSD ≤ 5 %). All Data reduction, including background corrections, limits of detections and mass count to ppm conversions were done using Iolite software (Paton et al.,2011).

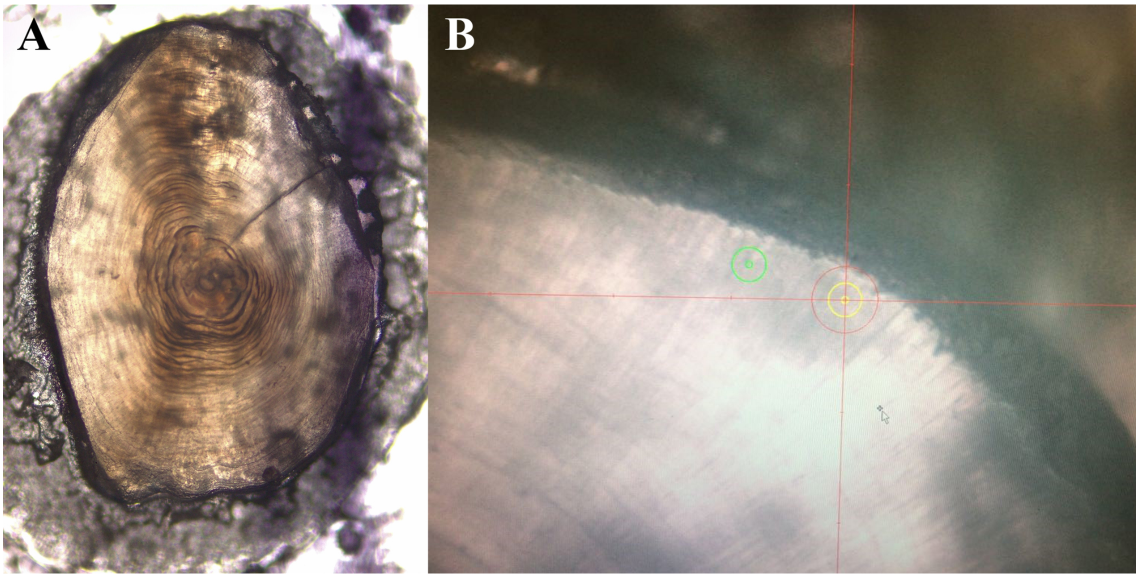


Figure 4 - A) Microphotograph of a *Solea senegalensis* sagittae otolith, highlighting the microstructure in terms of daily growth rings, and B) the peripheral area where sampling took place (laser diameter = 25 µm).

2.4 Data analyses

Otolith elemental concentration (Me) values (ppm) were converted to molar concentrations and standardized to calcium (Ca) ($Me:Ca_{Otolith}$), as they are likely to substitute for Ca in fish otoliths (Campana, 1999; Doubleday et al., 2014). All further data analyses were carried out on the $Me:Ca_{Otolith}$ data. The same procedure was adopted for the water samples ($Me:Ca_{Water}$). For each experimental condition, the mean values of $Me: Ca_{water}$, $Me: Ca_{otolith}$ were calculated using the three replicates.

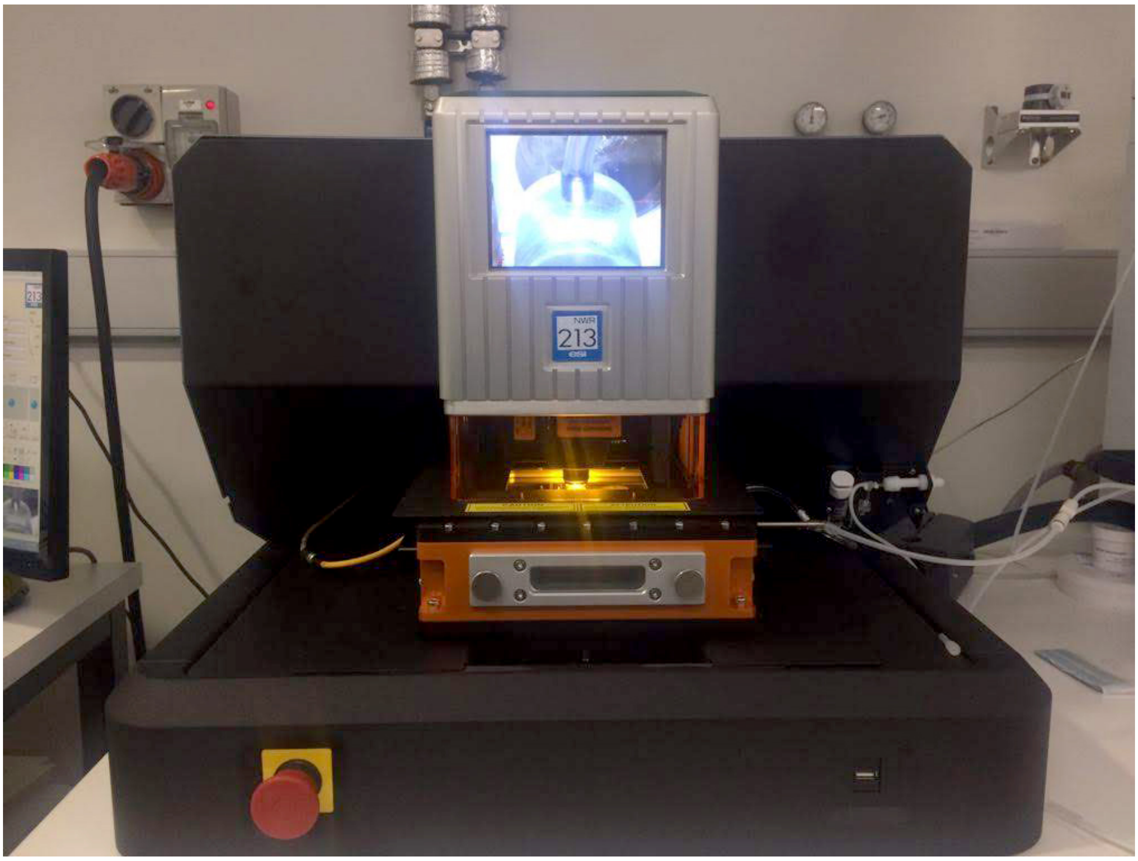
Differences in the elemental concentrations (Me:Ca) in the rearing conditions in water were analyzed using a Kruskal-Wallis chi-squared test, and whenever significant differences were found, multiple comparisons Dunn tests with Bonferroni adjustment were performed (R package dunn.text).

Variation in growth rates (total length, mm day^{-1}) per treatment were analyzed using generalized linear mixed models (GLMM) with a Gamma distribution (allowing only for > 0 estimates) (R package lme4), with salinity, temperature and their interaction treated as fixed categorical factors, and replicate aquariums treated as a random factor to remove the effect of the location of each aquarium in the experimental design.

Partition coefficients (D_{Me}) were also calculated for each experimental condition by dividing the relation Me:Ca in the otoliths by the Me:Ca in the water (Morse and Bender, 1990). D_{Me} provides an easy and useful metric to compare incorporation of elements in various treatments, since it is able to describe the chemical concentrations of otoliths in relation to the chemical concentration in the water, providing a useful tool to evaluate abiotic and biotic effects on otolith elemental incorporation and to perform comparisons between species and studies.

$$D_{Me} = \frac{Me:Ca_{otolith}}{Me:Ca_{water}}$$

Differences among treatments in $Me:Ca_{Otolith}$ and D_{Me} were analyzed separately using generalized linear mixed models (GLMM) with a Gamma distribution (R package lme4). Salinity, temperature and the interaction between them were treated as fixed categorical factors. Replicate aquaria were included as a random factor to remove the effect of the location of each aquarium in the experimental design. The validation of the model was done through plotting deviance residuals against fitted values and visual inspection of the absence of patterns in the data. The assessment of the significance of the fixed effects within the GLMM was done with Wald χ^2 tests (R package car), and whenever significant differences were found, multiple comparisons Tukey tests with Bonferroni adjustment were performed. Data analysis was performed in R software (R Core Team 2017) with packages lme4, car, multcomp, dunn.text, considering a significance level of 0.05 for all test procedures.



Chapter 3 – Results

3.1 Experimental conditions

Temperature and salinity reflected the desired experimental levels, with slight variations during the experimental period (Table 1). In addition, the element concentrations in the water varied with the different salinity regimes (Table 1): for instance, Sr increased in concentration with increasing salinity, while on the other hand, the influence of salinity was not so notable for Ba, which decreased only from salinity 5 to 18. Similarly to Sr, an increase in temperature corresponded to an increase in both Ba and Ca concentrations. While Mn was relatively stable in all treatments, for both ^{24}Mg and ^{25}Mg, an increase in salinity and temperature led to an increase in their concentration in the water.

Table 1- Summary of the experimental conditions of *Solea senegalensis* in each treatment, showing mean (and standard error) of water temperature (ºC), salinity, Sr, Ba, Mn, ^{24}Mg, ^{25}Mg and Ca concentrations (mgL-1).

Treatment		Salinity	Temperature (°C)	Sr (mgL^{-1})	Ba (mgL^{-1})	Mn (mgL^{-1})	^{24}Mg (mgL^{-1})	^{25}Mg (mgL^{-1})	Ca (mgL^{-1})
Temperature (°C)	Salinity								
16	5	5.047 ± 0.018	15.934 ± 0.012	2.526± 0.292	0.097± 0.000	0.019± 0.001	543.536± 114.272	516.696± 108.713	85.787± 16.765
	18	18.173 ± 0.040	15.913 ± 0.136	7.532± 0.248	0.026± 0.001	0.011± 0.001	1924.256± 88.551	1848.103± 84.016	266.137± 12.945
	30	30.041 ± 0.025	16.051 ± 0.133	10.939± 0.107	0.026± 0.022	0.018± 0.001	3053.577± 31.814	2938.625± 29.801	410.774± 4.990
21	5	5.017 ± 0.020	21.359 ± 0.804	2.840± 0.106	0.134± 0.021	0.014± 0.001	631.519± 29.638	599.847± 27.830	101.210± 4.733
	18	18.247 ± 0.039	20.990 ± 0.900	8.050± 0.174	0.059± 0.009	0.014± 0.001	2115.071± 74.900	2025.300± 70.513	292.176± 10.945
	30	30.056 ± 0.365	21.449 ± 0.950	11.452± 0.183	0.059± 0.010	0.012± 0.000	3259.035± 86.632	3132.445± 82.130	438.427± 13.173

Element to Ca ratios are useful indicators of comparison between elements, once they compete with Ca to enter in the otolith matrix and are presented in Table 2.

Although there was slight a tendency for Sr:Ca ratio decreasing with the increase of both salinity and temperature, there were no differences between treatments ($\chi^2_{(5)}$= 7.09, p =0.21). For Ba:Ca there were significant differences between treatments (χ^2_{5}= 14.2929, p =0.01) namely between 21ºC.5 salinity and 21ºC.30 salinity (p=0.0049). Likewise, for Mn:Ca ratio there were significant differences among treatments ($\chi^2_{(5)}$= 13.24, p =0.02), particularly between 21ºC.5 salinity and 21ºC.30 salinity (p=0.0202). For ^{24}Mg:Ca and ^{25}Mg:Ca there were no significant differences between treatments ($\chi^2_{(5)}$= 10.31, p =0.07).

Table 2 - Summary of the element to Ca ratios obtained in each experimental treatment.

Treatment		Sr:Ca (µmol mol $^{-1}$)	Ba:Ca (µmol mol $^{-1}$)	Mn:Ca (µmol mol $^{-1}$)	^{24}Mg:Ca (µmol mol $^{-1}$)	^{25}Mg:Ca (µmol mol $^{-1}$)
Temp (°C)	Salinity					
16	5	21343.54± 1796.14	85.44± 1.40	234.43 ± 57.22	12200706.71± 1245270.25	11899136.23± 1266892.41
	18	20257.89± 1916.92	61.60± 17.70	87.23 ± 35.93	13673164.16± 885354.25	13412216.071± 917744.26
	30	17979.51± 1680.60	38.73± 8.32	124.41± 9.25	13901023.03± 646505.52	13534651.26± 702045.5
21	5	18962.49± 1497.48	133.546± 12.183	418.68± 88.25	10297415.96± 271328.05	10023635.63± 303291.98
	18	17610.82± 1215.13	47.686± 3.737	105.70± 52.31	12566666.06± 37274.11	12207367.52± 103409.83
	30	15660.77± 896.28	28.027± 1.735	55.08± 9.88	13284304.94± 163613.63	12830303.14± 204629.73

3.2 Fraction of survivors and growth

During the experimental period, the fraction of survivors was high in all experimental conditions (Figure 5). The lowest survival rate was observed at 21°C and 5 salinity, with 3 dead individuals, corresponding to a fraction of survival of 0.85. At 16°C.5 salinity, 16°C. 30 salinity, 21°C. 18 salinity and 21°C. 30 salinity there was only one death per condition, resulting in slightly variable survival fraction due to the different number of individuals per condition (Table 3). In condition 16°C. 18 salinity no mortality was observed throughout the experiment.

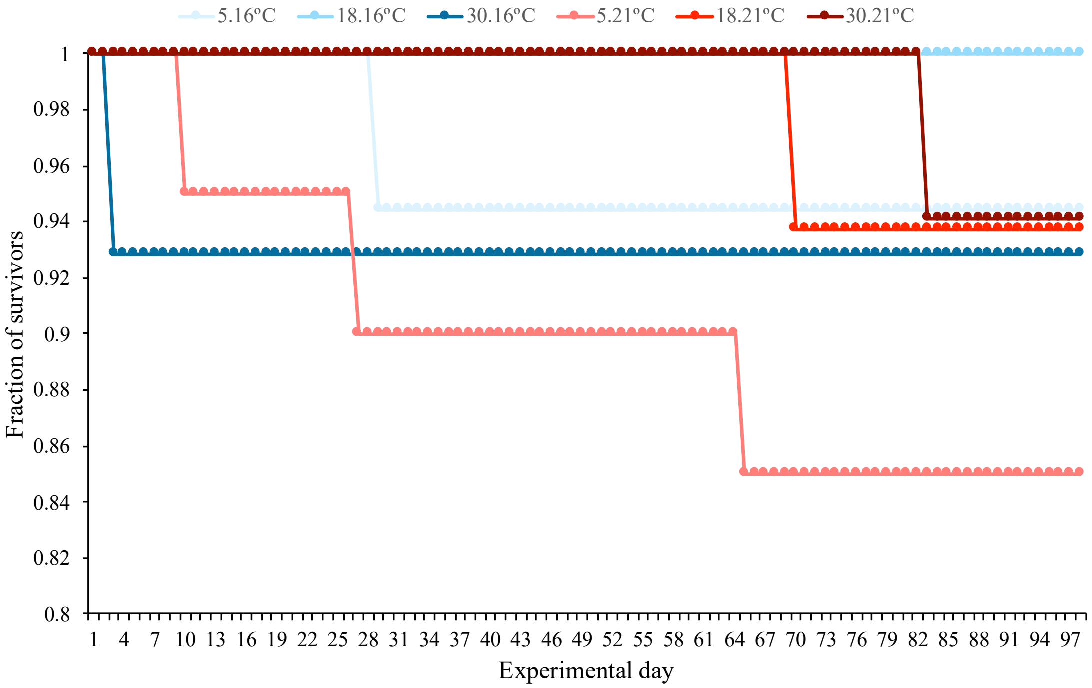


Figure 5- Daily fraction of survivors of juvenile *Solea senegalensis* for all the experimental treatments Each treatment is indicated by different colors.

No statistical differences were found in the estimated growth of juvenile *S. senegalensis* for the experimental treatments (Table A1). Still, there seems to be a tendency on the influence of experimental treatments on the growth (Table 3, Figure 6), particularly for fish exposed to 21ºC and salinity of 18, which had a mean growth of 1,95 cm. Increasing temperature seems to yield the capacity of increasing growth in the tested experimental conditions, although not statistically significant.

Table 3 - Summary of mean total length (and standard error) by treatment at the beginning and at the end of the experience.

Treatment		Start	End	Initial number of individuals per condition
Temperature (°C)	Salinity	Mean TL (cm)	Mean TL (cm)	
16	5	10.11 ± 0.24	11.47 ± 0.24	20
	18	9.63 ± 0.25	11.13 ± 0.25	20
	30	9.83 ± 0.22	10.83 ± 0.26	21
21	5	9.37 ± 0.22	10.82 ± 0.23	20
	18	9.64 ± 0.3	11.59 ± 0.31	18
	30	9.94 ± 0.25	11.47 ± 0.15	20

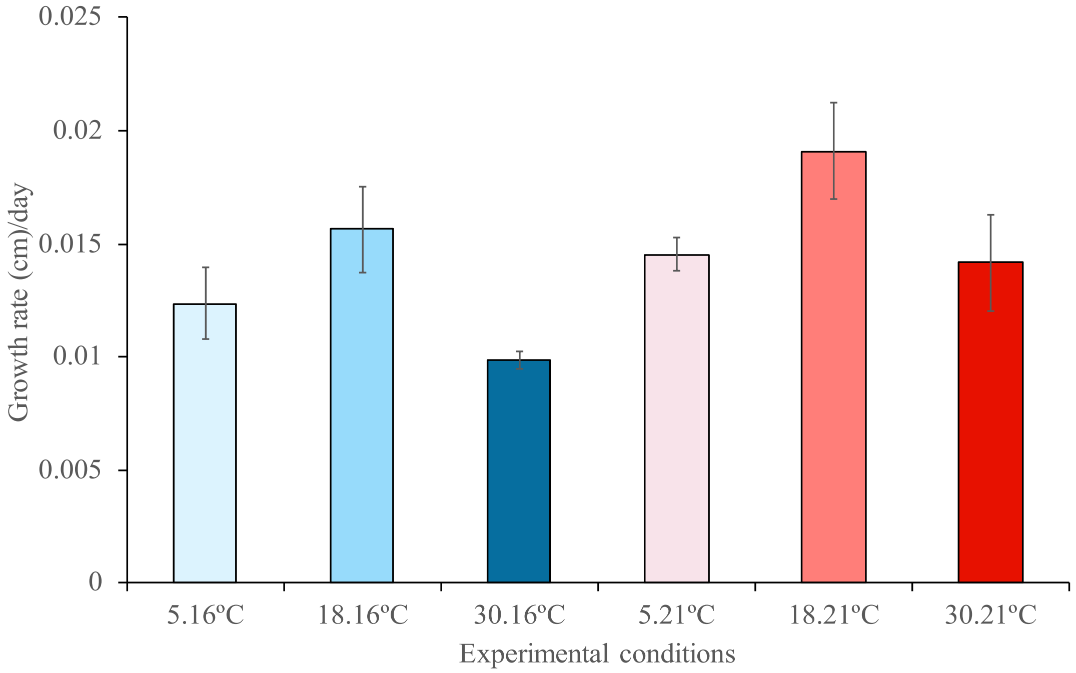


Figure 6- Mean and standard error of the growth rates for all experimental treatments of juvenile *Solea senegalensis*.

3.3 Otolith microchemistry

The elemental concentration in the otoliths varied significantly according to the different treatments (Table 4).

Table 4 - Mean and standard error of Sr, Ba, Mn, ^{24}Mg, ^{25}Mg and Ca concentrations (mgL^{-1}) in the otolith edge of *Solea senegalensis* in each treatment.

Treatment		Sr (mgL^{-1})	Ba (mgL^{-1})	Mn (mgL^{-1})	^{24}Mg (mgL^{-1})	^{25}Mg (mgL^{-1})	Ca (mgL^{-1})
Temperature (°C)	Salinity						
16	5	1970.40 ± 58.63	2.32 ± 0.13	2.8 ± 0.24	8.48 ± 0.55	8.02 ± 0.32	389172.55±512.44
	18	2183.10 ± 44.66	2.53 ± 0.14	2.63 ± 0.25	9.65 ± 0.71	8.32 ± 0.57	388385.29±373.60
	30	2430.54 ± 72.50	3.11 ± 0.15	2.33 ± 0.34	7.78 ± 0.46	9.06 ± 0.35	387868.75±1195.1
21	5	2096.29 ± 84.63	2.82 ± 0.22	1.97 ± 0.10	8.27 ± 0.48	7.81 ± 0.52	391007.29±436.00
	18	2025.43 ± 67.08	2.35 ± 0.11	3.17 ± 0.33	8.69 ± 0.39	8.4 ± 0.35	389976.67±424.18
	30	2257.44 ± 65.83	3.39 ± 0.24	2.41 ± 0.24	8.02 ± 0.48	8.72 ± 0.48	388840.63±659.88

Sr:Ca ratio in otoliths were significantly influenced by the salinity levels in the experimental treatments ($\chi^2_{(2)}$= 21.8894, p = 1.765 e^{-5}; Table A2; Figure 7). Higher Sr:Ca were observed at the highest salinity (30), and were significantly different from the lowest salinity treatments (5) (Tukey multiple-comparisons test with Bonferroni correction; 16ºC.5 salinity -16ºC.30 salinity (p=0.0076); for 21ºC.30 salinity - 16ºC. 5 salinity (p=0.0185) and for 20ºC. 5 salinity -16ºC. 30 salinity (p=0.0473) - Table A3). No interaction between temperature and salinity was found to influence the ratio of Sr:Ca (Table A3). In contrast, Sr:Ca in the water was lower at higher salinities, resulting in a linear negative relationship between the Sr:Ca in the water and in the otolith (Figure 8).

The Ba:Ca ratio increased with salinity (Figure 7), and a significant difference was observed between the higher salinity (30) and the lower salinities (5 and 18, respectively) ($\chi^2_{(2)}$= 29.2178, p = 4.523 e^{-7}; Table A2). In particular, differences were obtained between the conditions 16ºC. 30 salinity -16ºC.5 salinity (p=0.0087). 20ºC.30 salinity -16ºC.5 salinity (p=0.0216) and 20ºC.5 salinity -16.30 salinity (p=0.0250) (Table A4). No interaction between temperature and salinity was found to influence the

Ba:Ca ratio (Table A2). Similar to Sr:Ca, the Ba:Ca in the water was also lower at higher salinities, resulting in a negative non-linear relationship between the Ba:Ca in the water and in the otolith (Figure 8).

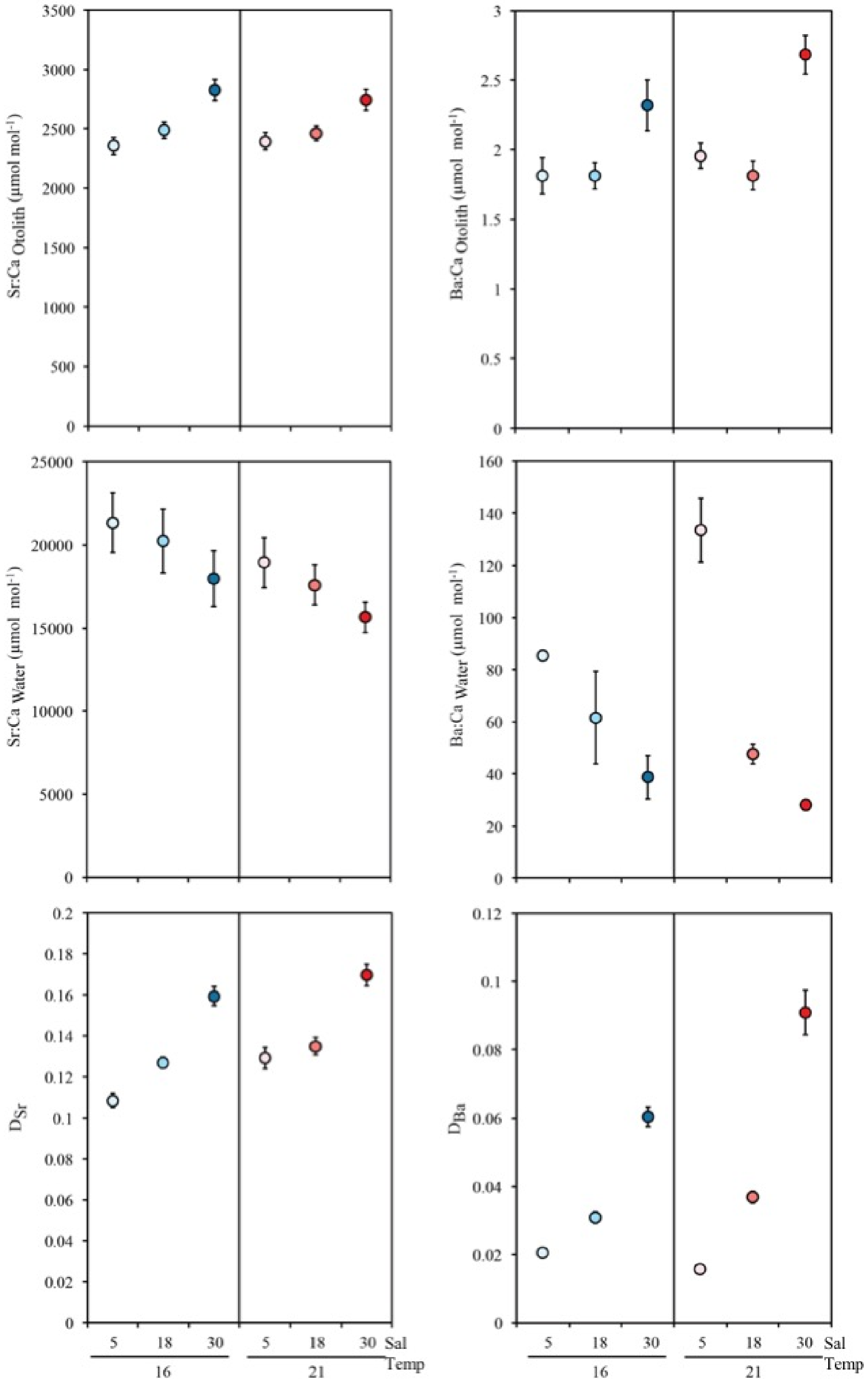


Figure 7- Mean and standard error of Sr: Ca$_{Otolith}$; Ba: Ca$_{Otolith}$; Sr: Ca$_{Water}$; Ba: Ca$_{Water}$; D$_{Sr}$ and D$_{Ba}$ across salinity (5, 18, 30) and temperature (16 and 21) experimental treatments of *Solea senegalensis*. Different treatments are indicated by different colors.

No influence of salinity and temperature (nor their interaction) was found in the ratios of Mn:Ca, ^{24}Mg:Ca, ^{25}Mg:Ca in juvenile *S. senegalensis* otoliths (Table A2).

$D_{Sr.}$ ranged between 0.11 ± 0.003 and 0.17 ± 0.005, with a higher incorporation of Sr at higher salinity and water temperature (Figure 7; Table A5). Wald χ^2 test detected an influence of salinity ($\chi^2_{(2)}$ =74.311, p=2.2 e^{-16}), temperature ($\chi^2_{(1)}$ =22.042, p=2.668 e^{-6}) and on the interaction between them ($\chi^2_{(5)}$=122.98, p=2.2 e^{-16}), and multiple-comparisons post hoc tests revealed significant differences between most treatments (Table A6).

D_{Ba} varied between 0.015± 0.001 and 0.09±0.006, and the highest value refers to the condition 30.21ºC (Fig. 7, Table A5). Significant differences were found for salinity ($\chi^2_{(2)}$ =233.499, p<2.2 e^{-16}), temperature ($\chi^2_{(1)}$ =87.238, p<2.2 e^{-16}) and also the interaction between them ($\chi^2_{(2)}$ =46.350, p =8.616 e^{-11}). The multiple comparisons test (Table 9) revealed differences between most treatments.

The D_{Mn} ranged between 3.25 e^{-6}±1.86 e^{-7} and 0.1±0.004 and presented significant differences for salinity ($\chi^2_{(2)}$ =98.362, p<2.2 e^{-16} (Table A5), and in the interaction between salinity and temperature ($\chi^2_{(2)}$ =52.236, p=5.54 e^{-12} (Table A5). Tukey test with Bonferroni correction indicated significant differences among the majority of treatments (Table A8).

D_{24Mg} ranged between 2.68 e^{-6} ± 1.83 e^{-7} and 3.52 e^{-6}± 2 e^{-7}, and presented significant differences for salinity ($\chi^2_{(2)}$ =25.3816, p=3.079 e^{-6}) and temperature ($\chi^2_{(1)}$ =8.2283, p=0.004124) (Table A5). There were differences between 5 of the 15 combinations tested, mainly between salinities 5 and 30, but also between 18.21ºC - 30.16ºC, 30.16ºC - 18.16ºC (Table A9). D_{25Mg} ranged between 0.009 ± 0.0006 and 0.079 ± 0.01 and presented only significant differences in salinity 30 (Wald χ^2 Test confirmed that only salinity was significant; $\chi^2_{(2)}$ =11.5097, p=0.003167) (Table A5).

The Tukey test with Bonferroni correction indicated significant differences between 5.21 - 30.16 (Table A10).

A negative linear trend was observed for the relationship between Sr:Ca$_{\text{otolith}}$ and Sr:Ca$_{\text{water}}$, and between D$_{\text{Sr}}$ and Sr:Ca$_{\text{water}}$, while non-linear negative relationships were obtained between Ba:Ca$_{\text{otolith}}$ and Ba:Ca$_{\text{water}}$, and D$_{\text{Ba}}$ and Ba:Ca $_{\text{water}}$ (Figure 8).

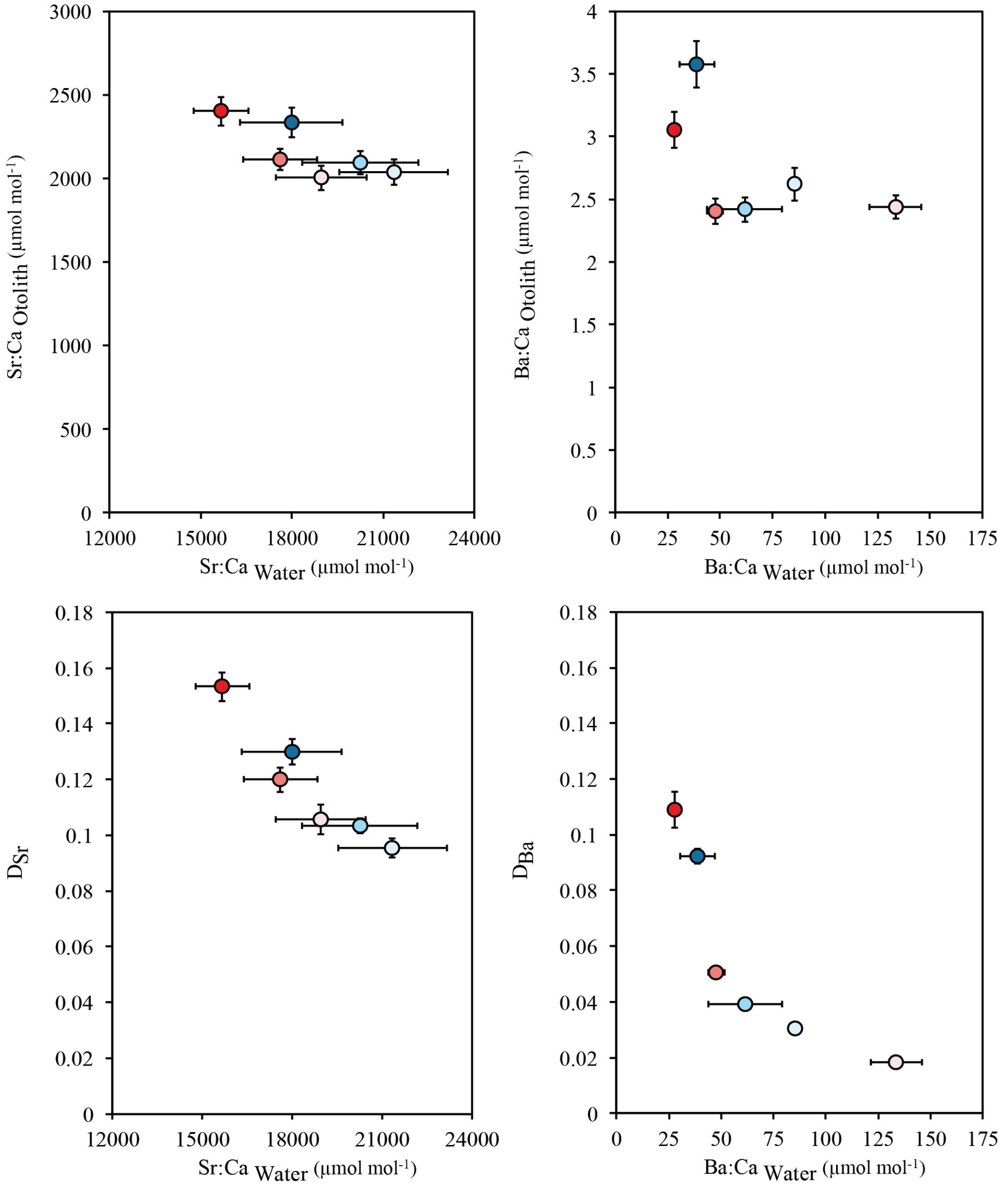
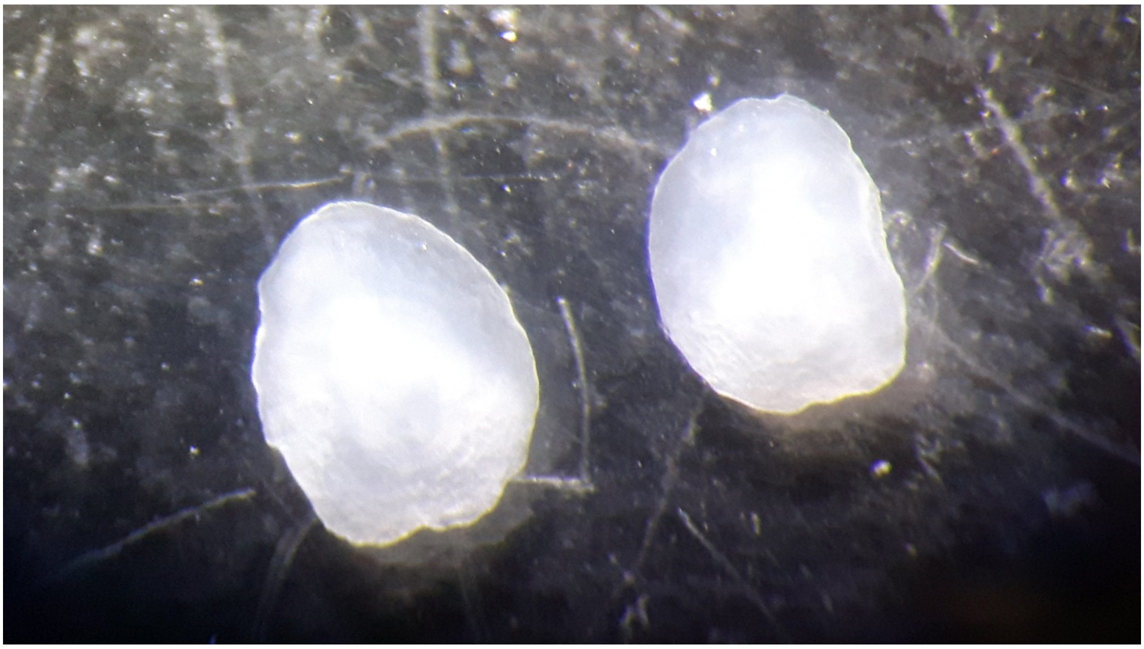


Figure 8- Mean and standard error of relationship between Sr: Ca$_{\text{otolith}}$ x Sr: Ca$_{\text{water}}$, Ba: Ca$_{\text{otolith}}$ x Ba: Ca$_{\text{water}}$, D$_{\text{Sr}}$ x Sr: Ca $_{\text{water}}$ and D$_{\text{Ba}}$ x Ba: Ca $_{\text{water}}$. Different treatments are indicated by different colors.



Chapter 4 – Discussion

Variation of the elemental composition in otoliths in relation with the environment is a remarkably useful tool for determining the fish movements, their migration routes and habitat use patterns. In this study, we performed a controlled laboratory experiment to evaluate the effects of variations in water salinity and temperature on the elemental composition of *Solea senegalensis* otoliths, a species with high economic importance (fisheries, aquaculture) that performs migrations between marine and brackish waters, mainly during the first year of life (Primo et al., 2013; Tanner et al., 2013; Vinagre, et al., 2008).

Despite reflecting the desired combination of salinity (5, 18 and 30) and water temperature (16 and 21ºC), the experimental conditions did not influence significantly growth in juvenile *S. senegalensis*, as we did not find differences between treatments during the experiment. Still, there was a tendency for higher growth rates in the treatments at 18 salinity in both temperature conditions (16 and 21ºC). These are the conditions that juvenile Senegalese sole will most typically face within estuaries during their ontogenic migrations (e.g. Able, 2005; Cabral et al., 2007; Cyrus & Martin, 1991; Martinho et al., 2007; Rijnsdorp et al., 1985), which favour a rapid growth and survival. Overall, relatively low growth rates were obtained, which can be due to the stress induced by the experimental conditions stress may also directly affect the incorporation rate of the various elements in the otolith; still, survival along the 98-day trial was always high, ensuring the robustness of the data analyses.

Previous studies have highlighted a positive relationship between water chemistry and otolith elemental composition in both fresh and saltwater (Bath et al., 2000; Elsdon & Gillanders, 2003; Kraus & Secor, 2004; Miller, 2009), underlining the usefulness of this technique to unravel fish migrations and habitat use patterns. In this work, the influence of temperature and salinity on the elemental composition of the otoliths was also verified, with the elemental composition of the water as the main driver of the elemental composition of the otoliths, in agreement with other authors (e.g. Elsdon & Gillanders, 2002, 2004; Martin et al., 2004; Martin & Wuenschel, 2006). In more detail, when we analyzed the Me:Ca ratio in the otoliths, no differences were obtained among treatments for ^{24}Mg:Ca, ^{25}Mg:Ca, Mn:Ca, while Sr:Ca and Ba:Ca both distinguished between the lower and higher salinities (5 and 30, respectively), mainly at 21ºC, strengthening the use of Sr and Ba as key indicators of habitat use for marine (Sr) and freshwater (Ba) habitats.

Analyzing the Mn:Ca, ^{24}Mg:Ca and ^{25}Mg:Ca in the otoliths, there were no significant differences between treatments. Tanner et al. (2012) also found no differences in Mn:Ca otolith concentration regardless of where the fish were obtained along a wide geographical range, and even between the otolith zones (core or edge). Metabolic effects may play a greater role in Mn incorporation than environmental factors (Limburg et al., 2015; Tanner et al., 2012), rather than just relying on the environmental availability. According to Elsdon & Gillanders (2003), Mn is an important element that seems to be regulated by fish since it is used in metabolic processes and can become toxic, being essential to keep it at a constant concentration. However, the same authors, citing Pentreath, 1976, argue that there is some evidence that Mn from otoliths originates from the diet or the food chain, adding a confounding effect for the successful use of this element as a natural tag for elucidating habitat use patterns.

In parallel with Mn, no differences between treatments were also observed for both ^{24}Mg:Ca and ^{25}Mg:Ca, as also observed in the review by Sturrock et al. (2012). This element is correlated with fish growth rates (Sturrock et al. 2015) , and is required for many metabolic pathways, namely growth. Hence, the high variability found in

Mg:Ca can be explained, according to Grammer et al. (2017), due to individual metabolic and growth processes. Even so, changes in environmental conditions may be reflected in changes in the assimilation rates of this element, which could not be demonstrated in this work.

Despite that salinity had a positive influence on Sr:Ca $_{\text{otolith}}$, no temperature effects were detected on Sr:Ca $_{\text{otolith}}$. Indeed, salinity is known to influence on Sr:Ca on fish otoliths, as observed elsewhere (e.g Elsdon & Gillanders, 2002, 2004; Miller, 2011; Reis-Santos et al., 2013), being an indicator of marine environments. In this work, we only observed differences in Sr:Ca between near freshwater (5) and marine-like (30) salinities, which corroborates the usefulness of using the increase in Sr as an indicator of the transition between fresh and saltwater. However, this also reveals some potential drawbacks in brackish water (such as in our mid-estuary simulation, 18 salinity), due to the high variability of this element found in these waters. We also did not observe any significant effects of temperature on Sr incorporation in the otolith matrix of juvenile Senegalese sole, in agreement with Elsdon & Gillanders (2004). Still, the influence of temperature on Sr incorporation has been reported by several other papers based on experimental work, in which temperature had either a positive (see Bath et al. (2000); Reis-Santos et al. (2013) or a negative (Townsend et al., 1995) relationship with Sr in the otoliths.

Similarly to Sr:Ca, Ba:Ca $_{\text{otolith}}$ ratio also increased with salinity, but with more accountable variability in the otoliths. Notwithstanding that the main source of Ba is environmental, due to variations in water temperature or salinity and the surrounding water, which can affect its uptake into the otolith matrix (Bath et al., 2000; Elsdon & Gillanders, 2004), other factors may also play an important role, such as diet and growth rates (Grammer et al., 2017). This element has been used typically in combination with

Sr to unravel environmental and migratory histories (Bath et al., 2000; de Vries et al., 2005; Elsdon & Gillanders, 2002; Reis-Santos et al., 2013). However, our results also reveal some potential drawbacks in brackish water, as is the case with Sr (such as in our mid-estuary simulation, 18 salinity), due to the high variability of this element found in these waters. It is still unclear how water temperature influences Ba:Ca $_{\text{otolith}}$, since other authors (e.g. Elsdon & Gillanders 2002, 2004; Miller 2009) found a positive effect of temperature on Ba uptake, while others (e.g. Bath et al., 2000; Martin & Wuenschel 2006) reported the absence of effects, leading to the interpretation that there might be species-specific patterns of element uptake and incorporation in the otolith.

Our partition coefficients for Sr and Ba (D_{Sr} and $D_{Ba,\ \text{respectively}}$) were the best metrics for comparing the relation between elemental concentrations in the otoliths and in the water (as in Miller, 2009), since it becomes more noticeable that the incorporation rather than partition coefficient was influenced by temperature and salinity (Reis-Santos et al., 2013). The low values found in all partition coefficients can be justified by the slow otolith precipitation rate in the experimental aquaria, that is to say that despite good discrimination between freshwater and salt water with these D_{Me} values, if habitat changes are substantially rapid, this tool cannot be used to detect them (Mucci & Morse, 1983), added to the many physiological boundaries by which elements must pass through before being incorporated in the otolith matrix (Campana, 1999).

The differences observed in D_{Sr} between treatments were higher than those obtained for Sr:Ca $_{\text{otolith}}$. In fact, D_{Sr} presented differences in both salinity and temperature, and despite showing the same trend as for Sr:Ca $_{\text{otolith}}$, in this case is more pronounced as verified by other authors (Kraus & Secor, 2004; Reis-Santos.et al., 2013). These results highlight the relationship between the elemental composition of water and otoliths, and that the experimental treatments actually featured these differences.

For D_{Ba} there were differences not only in salinity as for Ba:Ca $_{otolith}$, but also in temperature, and an interaction between salinity and temperature. D_{Ba}, as well as other partition coefficients, express the relation of the elemental concentration in the otolith to the elemental concentration in the water; hence, we can observe that the rate of incorporation is influenced by salinity, temperature and the interaction between them. Relationship between the incorporation varies differently for different combinations of temperature and salinity. This interaction between salinity and temperature makes interpreting the results more complex, which may hamper our attempts to identify patterns of habitat use and/or migrations (Reis-Santos et al., 2013). Within each treatment, the decrease of Ba:Ca $_{Water}$ along the salinity gradient must have resulted in a greater elemental incorporation (Reis-Santos et al., 2013); in this scope, several authors have related D_{Ba} with elemental concentration in water and argued that greater incorporation takes place at lower concentrations in the water, suggesting that more Ba is proportionally incorporated in lower water concentrations (Bath et al., 2000; de Vries et al., 2005; Reis-Santos et al., 2013).

Despite that no differences were observed in the Mn:Ca$_{otolith}$, D_{Mn} had a different behavior in relation to the Mn:Ca $_{otolith}$, as the interaction between salinity and temperature was significant for the D_{Mn}. Since the elemental concentration in otolith did not show significant differences between treatments, and in D_{Mn} there is an interaction between salinity and temperature, then this difference probably originates in differences in elemental concentration in water, as observed, but that were not translated into the otoliths. While $D^{24}{}_{Mg}$ showed differences for salinity and temperature, $D^{25}{}_{Mg}$ only showed differences for salinity perhaps due to the high variability found in the otoliths for this element. In elements with high physiological influence, such as ^{24}Mg, ^{25} Mg and Mn, the use of the partition coefficient may be misleading since physiological processes (e.g., growth and development of gonads) can modify the composition of the blood and consequently of the otolith chemistry. Further research on the underlying mechanisms of ion transport in fish is clearly needed to address this topic.

In our case, there was no effect of temperature on Sr:Ca, Ba:Ca, Mn:Ca, ^{24}Mg:Ca and ^{25}Mg:Ca in otoliths. This can be due to the fact that these temperatures are within the range they inhabit typically in estuaries and coastal areas, and hence there is no effect on the uptake and rates of incorporation of elements on the otolith matrix. Another possible cause is that it is possible that temperature may influence the concentration of Sr: Ca differently in different species (Collingsworth et al., 2010).

Contrary to the literature, in which has been demonstrated that the concentrations of Sr:Ca $_{\text{otoliths}}$ follow the same trend as the elemental concentration of water (e.g. Elsdon & Gillanders, 2003; Reis-Santos et al., 2013), in our case Sr:Ca $_{\text{otolith}}$ increased with salinity, while Sr:Ca $_{\text{water}}$ decreased, resulting in a negative linear relationship between Sr:Ca $_{\text{otolith}}$ and Sr:Ca $_{\text{water}}$. Since the species in study realizes ontogenic migrations along the river – estuary – sea continuum, the Sr:Ca $_{\text{water}}$ gradient is the most important consideration in the interpretation of Sr:Ca $_{\text{otolith}}$ variability (Kraus & Secor 2004). The concentration of Sr: Ca $_{\text{water}}$ and Ba: Ca $_{\text{water}}$ decreases with the increase of the salinity, this can be explained by the increase of the uptake with the salinity. This is true since the partition coefficient also increases with salinity. In Izzo et al. (2018), the interactive model of salinity and temperature showed no differences in temperature and salinity for Sr:Ca$_{\text{otolith.}}$ We also did not observe the effect of temperature, contrary to the literature (Elsdon & Gillanders 2002; Miller 2011). Besides the variations along the estuary due to daily tidal movements, it is known that the temperature can also be an important factor in the incorporation of Sr in the otolith matrix (Kraus & Secor, 2004).

For the Ba: Ca $_{\text{otolith}}$ and Ba: Ca $_{\text{water}}$, and contrary to Sr: Ca $_{\text{otolith}}$ and Sr: Ca $_{\text{water}}$, a non-linear negative relationship was observed, which can be explained by the addition of freshwater to maintain a constant salinity throughout the experiment. In this way, some variability in Ba levels could have been introduced by this procedure, as was also referred in Nims (2012). Previous studies showed a positive linear relationship between Ba:Ca $_{\text{water}}$ and Ba:Ca $_{\text{otolith}}$ (Miller, 2009), which is in contradiction with our results, highlighting once again the species-specific patterns in element uptake, combined with different basal levels in the water and their temporal and spatial variability. In fact, the variation of Ba:Ca $_{\text{otolith}}$ may indicate that changes in other environmental variables may influence the relationship between the elemental concentration of calcified structures and water (Bath et al., 2000; Elsdon & Gillanders, 2003).

The decreasing relationship found between D_{Sr} and Sr: Ca $_{\text{water}}$ and between D_{Ba} and Ba: Ca $_{\text{water}}$ indicates that elemental concentrations in the water were still far from stabilizing, thus ensuring that elemental concentrations in water were representative of those found in the field. This relationship is similar to that obtained by Bath et al. (2000) in *Leiostomus xanthurus* also in a laboratory experiment, and shows that the degree of discrimination that occurs between water and otoliths is probably concentration dependent (Elsdon & Gillanders, 2003), which tends to stabilize at high ambient element concentrations.

For *Solea senegalensis*, there was no effect of the interaction between salinity and temperature on the elemental composition in the otolith, as was not observed for other species as well (e.g. Chesney et al., 1998; Martin et al., 2004; Martin & Wuenschel, 2006; Reis-Santos et al., 2013). However Elsdon & Gillanders (2002) showed a significant effect of this interaction, which is supposed to take place in natural conditions. Understanding how salinity influences elemental otolith concentration can be a challenging task since by altering salinity the elemental concentration is also altered, hampering our understanding of where the direct influence of salinity or elemental concentration takes place. Moreover, and apart from salinity, temperature and ambient elemental concentration, other biological factors play a role in determining the elemental concentrations in the otolith, such as genetic profiles, sex and age (Grammer et al., 2017; Izzo et al., 2018; Reis-Santos et al., 2013). Still, we tried to control for these effects by using juvenile fish obtained from a hatchery, with similar age and length distributions.

In summary, the elemental composition of the otolith results from several environmental factors, supporting the assumption that variations in the environment are reflected in the composition of the otolith. Knowing that Sr and Ba are more influenced by the concentration of elements in water and that Mn, on the other hand, seems to be less influenced by environmental concentrations, these elements can be used to respond to different ecological issues. Since Mn, ^{24}Mg and ^{25}Mg are more affected by physiological factors, then Sr and Ba are more suitable for the reconstruction of fish movements along the concentration gradients (Elsdon & Gillanders, 2003). In addition, it would be worth considering the species-specific patterns of element uptake before proceeding to field studies with broader scientific objectives (Reis-Santos et al., 2013).

Further work should combine laboratory experiments with species-specific information on the elemental incorporation of otoliths and field experiments, so as to better understand the processes involved in the incorporation of elements in the otolith matrix under natural conditions and the timeframe between element uptake and otolith assimilation. It may also be useful to include additional markers such as stable isotopes

(Walther & Thorrold, 2009; Woodcock & Walther, 2014) that function as a record of environmental conditions to facilitate the interpretation of patterns of use and movement of different fish species along salinity gradients.

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Appendix

Table A1 – Estimates of the fixed effects for growth rates, obtained by the GLMM analysis.

Fixed effects	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	91.329	19.921	4.584	$4.55e^{-06}$
Sal18	-13.907	26.786	-0.519	0.604
Sal30	5.226	30.091	0.174	0.862
Temp21	-18.86	26.52	-0.711	0.477
Sal18: Temp21	7.97	37.606	0.212	0.832
Sal30: Temp21	14.442	40.463	0.357	0.721

Table A2- Estimated fixed effects of the GLMM, examining the effects of salinity and temperature on Sr: Ca, Ba: Ca, Mn: Ca, ^{24}Mg: Ca, ^{25}Mg: Ca in the otolith of juvenile *Solea senegalensis*. *p<0.05; **p <0.01; ***p<0.001.

Me: Ca	Fixed effects	Estimate	Std. Error	t value	Pr(>|z|)
Sr: Ca	(Intercept)	4.98E-04	1.59E-05	31.316	< 2e-16 ***
	Sal18	-2.28E-05	2.23E-05	-1.023	0.306105
	Sal30	-8.04E-05	2.34E-05	-3.442	0.000578 ***
	Temp20	-6.05E-06	2.28E-05	-0.266	0.790511
	Sal18:Temp20	1.30E-05	3.21E-05	0.404	0.685955
	Sal30:Temp20	1.73E-05	3.21E-05	0.54	0.589523
Ba:Ca	(Intercept)	0.408996	0.023904	17.11	<2e-16 ***
	Sal18	0.008036	0.034201	0.235	0.8142
	Sal30	-0.08076	0.034084	-2.369	0.0178 *
	Temp20	-0.026294	0.033419	-0.787	0.4314
	Sal18:Temp20	0.020389	0.048616	0.419	0.6749
	Sal30:Temp20	-0.022386	0.04524	-0.495	0.6207
Mn:Ca	(Intercept)	0.39006	0.03893	10.02	<2e-16 ***
	Sal18	-0.03693	0.05402	-0.684	0.4942
	Sal30	0.06851	0.07034	0.974	0.3301
	Temp20	0.1049	0.06233	1.683	0.0924 .
	Sal18:Temp20	-0.12828	0.08144	-1.575	0.1152
	Sal30:Temp20	-0.12147	0.09635	-1.261	0.2074
^{24}Mg:Ca	(Intercept)	0.09824	0.004648	21.135	<2e-16 ***
	Sal18	-0.010178	0.006423	-1.585	0.113
	Sal30	0.014449	0.008022	1.801	0.0717 .
	Temp20	-0.001816	0.006647	-0.273	0.7847
	Sal18:Temp20	0.009694	0.009344	1.037	0.2995
	Sal30:Temp20	-0.012687	0.010491	-1.209	0.2265

^{25}Mg:Ca	(Intercept)	0.120186	0.007832	15.345	<2e-16 ***
	Sal18	-0.01751	0.010633	-1.647	0.0996 .
	Sal30	0.009905	0.013097	0.756	0.4495
	Temp20	-0.000898	0.011261	-0.08	0.9364
	Sal18:Temp20	0.017769	0.015714	1.131	0.2581
	Sal30:Temp20	-0.006073	0.017467	-0.348	0.7281
	Sal30:Temp20	-0.006073	0.017467	-0.348	0.7281

Table A3 Results of multiple comparisons, examining the effects of salinity and temperature on Sr:Ca in the otolith of *Solea senegalensis*.*p<0.05; **p <0.01; ***p<0.001.

	Estimate Std.	Estimate	Std. Error	Pr(>|z|)
18.16 - 5.16	$-2.278e^{-05}$	$2.225e^{-05}$	-1.023	0.91014
30.16 - 5.16	$-8.038e^{-0}5$	$2.336e^{-05}$	-3.442	0.00759**
5.20 - 5.16	$-6.050e^{-06}$	$2.278e^{-05}$	-0.266	0.99982
18.20 - 5.16	$-1.584e^{-05}$	$2.288e^{-05}$	-0.692	0.98286
30.20 - 5.16	$-6.912e^{-0}5$	$2.169e^{-05}$	-3.187	0.01805*
30.16 - 18.16	$-5.761e^{-05}$	$2.314e^{-05}$	-2.489	0.12699
5.20 - 18.16	$1.672e^{-05}$	$2.256e^{-05}$	0.742	0.97669
18.20 - 18.16	$6.941e^{-06}$	$2.266e^{-05}$	0.306	0.99964
30.20 - 18.16	$-4.635e^{-05}$	$2.146e^{-05}$	-2.160	0.25649
5.20 - 30.16	$7.433e^{-05}$	$2.364e^{-05}$	3.144	0.02063*
18.20 - 30.16	$6.455e^{-05}$	$2.374e^{-05}$	2.719	0.07133
30.20 - 30.16	$1.126e^{-05}$	$2.260e^{-05}$	0.498	0.99624
18.20 - 5.20	$-9.784e^{-06}$	$2.317e^{-05}$	-0.422	0.99829
30.20 - 5.20	$-6.307e^{-05}$	$2.200e^{-05}$	-2.867	0.04737 *
30.20 - 18.20	$-5.329e^{-05}$	$2.211e^{-05}$	-2.410	0.15219

Table A4- Results of multiple comparisons. examining the effects of salinity and temperature on Ba: Ca in the otolith of *Solea senegalensis*.*p<0.05; **p <0.01; ***p<0.001.

	Estimate	Std. Error	z value	Pr(>|z|)
18.16 - 5.16	-2.28E-05	2.23E-05	-1.023	1
30.16 - 5.16	-8.04E-05	2.34E-05	-3.442	0.00867 **
5.20 - 5.16	-6.05E-06	2.28E-05	-0.266	1
18.20 - 5.16	-1.58E-05	2.29E-05	-0.692	1
30.20 - 5.16	-6.91E-05	2.17E-05	-3.187	0.02157 *
30.16 - 18.16	0 -5.761e-05	2.31E-05	-2.489	0.19194
5.20 - 18.16	1.67E-05	2.26E-05	0.742	1

18.20 - 18.16	0 6.941e-06	2.27E-05	0.306	1
30.20 - 18.16	0 -4.635e-05	2.15E-05	-2.16	0.46184
5.20 - 30.16	7.43E-05	2.36E-05	3.144	0.02500 *
18.20 - 30.16	0 6.455e-05	2.37E-05	2.719	0.09834 .
30.20 - 30.16	0 1.126e-05	2.26E-05	0.498	1
18.20 - 5.20	-9.78E-06	2.32E-05	-0.422	1
30.20 - 5.20	-6.31E-05	2.20E-05	-2.867	0.06215 .
30.20 - 18.20	0 -5.329e-05	2.21E-05	-2.41	0.23901

Table A5- Results of fixed effects. examining the effects of salinity and temperature on D_{Sr}, D_{Ba}, D_{Mn}, D_{24Mg}, D_{25Mg} in the otolith of *Solea senegalensis*.*p<0.05; **p <0.01; ***p<0.001.

D_{Me}	Fixed effects	Estimate	Std. Error	t value	Pr(>|z|)
D_{Sr}	(Intercept)	10.6307	0.3263	32.58	< 2e-16 ***
	Sal18	-1.0171	0.446	-2.28	0.0226 *
	Sal30	-3.1278	0.4467	-7	2.51e-12 ***
	Temp21	-1.7911	0.4377	-4.09	4.28e-05 ***
	Sal18:Temp21	0.664	0.6067	1.09	0.2738
	Sal30: Temp21	1.001	0.5851	1.71	0.0871 .
D_{Ba}	(Intercept)	35.03	1.903	18.409	< 2e-16 ***
	Sal18	-9.421	2.357	-3.997	6.42e-05 ***
	Sal30	-22.345	2.072	-10.784	< 2e-16 ***
	Temp21	-20.246	2.075	-9.757	< 2e-16 ***
	Sal18:Temp21	14.237	2.743	5.19	2.11e-07 ***
	Sal30:Temp21	15.397	2.274	6.771	1.28e-11 ***
D_{Mn}	(Intercept)	91.286	8.019	11.384	< 2e-16 ***
	Sal18	-60.194	8.734	-6.892	5.50e-12 ***
	Sal30	-36.237	10.306	-3.516	0.000438 ***
	Temp21	115.936	19.903	5.825	5.70e-09 ***
	Sal18:Temp21	-112.168	20.568	-5.454	4.94e-08 ***
	Sal30:Temp21	-146.561	21.156	-6.928	4.28e-12 ***
D_{24Mg}	(Intercept)	1198620	56788	21.107	< 2e-16 ***
	Sal18	4832	81901	0.059	0.95295
	Sal30	368724	105604	3.492	0.00048 ***
	Temp21	-205577	76476	-2.688	0.00719 **
	Sal18:Temp21	207659	114032	1.821	0.06860 .
	Sal30:Temp21	-57480	133139	-0.432	0.66594
D_{25Mg}	(Intercept)	1430096	93212	15.342	<2e-16 ***
	Sal18	-54968	131163	-0.419	0.6752

Sal30	333248	166336	2.003	0.0451 *
Temp21	-234174	126798	-1.847	0.0648.
Sal18:Temp21	318024	186113	1.709	0.0875 .
Sal30: Temp21	50442	214056	0.236	0.8137

Table A6- Results of Tukey multiple comparison test with Bonferroni correction, examining the effects of salinity and temperature on D_{Sr} in the otolith of *Solea senegalensis*.*p<0.05; **p <0.01; ***p<0.001

Fixed effect	Estimate	Std. Error	z value	Pr(>|z|)
18.16 - 5.16	-1.02E+00	0.446	-2.28	0.338906
30.16 - 5.16	-3.13E+00	0.4467	-7.003	3.77e-11 ***
5.21 - 5.16	-1.79E+00	0.4377	-4.092	0.000642 ***
18.21 - 5.16	-2.14E+00	0.4365	-4.913	1.35e-05 ***
30.21 - 5.16	-3.92E+00	0.4052	-9.67	< 2e-16 ***
30.16 - 18.16	-2.11E+00	0.4307	-4.9	1.44e-05 ***
5.21 - 18.16	-7.74E-01	0.4215	-1.836	0.994334
18.21 - 18.16	-1.13E+00	0.4202	-2.683	0.109599
30.21 - 18.16	-2.90E+00	0.3876	-7.485	1.07e-12 ***
5.21 - 30.16	1.34E+00	0.4221	3.167	0.023116 *
18.21 - 30.16	9.84E-01	0.4208	2.338	0.291197
30.21 - 30.16	-7.90E-01	0.3883	-2.035	0.627965
18.21 - 5.21	-3.53E-01	0.4113	-0.858	1
30.21 - 5.21	-2.13E+00	0.3779	-5.627	2.74e-07 ***
30.21 - 18.21	-1.77E+00	0.3765	-4.711	3.69e-05 ***

Table A7-Results of multiple comparison. examining the effects of salinity and temperature on D_{Ba} in the otolith of *Solea senegalensis*. *p<0.05; **p <0.01; ***p<0.001

Fixed effects	Estimate	Std. Error	z value	Pr(>|z|)
18.16 - 5.16	-1.0171	0.446	-2.28	0.338906
30.16 - 5.16	-3.1278	0.4467	-7.003	3.77e-11 ***
5.21 - 5.16	-1.7911	0.4377	-4.092	0.000642 ***
18.21 - 5.16	-2.1442	0.4365	-4.913	1.35e-05 ***
30.21 - 5.16	-3.9179	0.4052	-9.67	< 2e-16 ***
30.16 - 18.16	-2.1107	0.4307	-4.9	1.44e-05 ***
5.21 - 18.16	-0.774	0.4215	-1.836	0.994334
18.21 - 18.16	-1.1271	0.4202	-2.683	0.109599
30.21 - 18.16	-2.9008	0.3876	-7.485	1.07e-12 ***
5.21 - 30.16	1.3368	0.4221	3.167	0.023116 *
18.21 - 30.16	0.9837	0.4208	2.338	0.291197
30.21 - 30.16	-0.7901	0.3883	-2.035	0.627965

18.21 - 5.21	-0.3531	0.4113	-0.858	1
30.21 - 5.21	-2.1268	0.3779	-5.627	2.74e-07 ***
30.21 - 18.21	-1.7737	0.3765	-4.711	3.69e-05 ***

Table A8-Results of multiple comparison. examining the effects of salinity and temperature on D_{Mn} in the otolith of *Solea senegalensis*. *p<0.05; **p <0.01; ***p<0.001

Fixed effects	Estimate	Std. Error	z value	Pr(>|z|)
18.16 - 5.16	-60.194	8.734	-6.892	8.25e-11 ***
30.16 - 5.16	-36.237	10.306	-3.516	0.006569 **
5.21 - 5.16	115.936	19.903	5.825	8.56e-08 ***
18.21 - 5.16	-56.426	8.902	-6.339	3.47e-09 ***
30.21 - 5.16	-66.862	8.594	-7.78	1.10e-13 ***
30.16 - 18.16	23.957	7.341	3.263	0.016518 *
5.21 - 18.16	176.13	18.542	9.499	< 2e-16 ***
18.21 - 18.16	3.768	5.189	0.726	1
30.21 - 18.16	-6.668	4.641	-1.437	1
5.21 - 30.16	152.173	19.332	7.872	5.33e-14 ***
18.21 - 30.16	-20.189	7.54	-2.678	0.111241
30.21 - 30.16	-30.625	7.174	-4.269	0.000295 ***
18.21 - 5.21	-172.362	18.621	-9.256	< 2e-16 ***
30.21 - 5.21	-182.798	18.476	-9.894	< 2e-16 ***
30.21 - 18.21	-10.435	4.949	-2.108	0.524832

Table A9-Results of multiple comparisons. examining the effects of salinity and temperature on D_{24Mg} in the otolith of *Solea senegalensis*. *p<0.05; **p <0.01; ***p<0.001

Fixed effects	Estimate	Std. Error	z value	Pr(>|z|)
18.16 - 5.16	4832	81901	0.059	1
30.16 - 5.16	368724	105604	3.492	0.00720 **
5.21 - 5.16 =	-205577	76476	-2.688	0.10779
18.21 - 5.16	6914	83046	0.083	1
30.21 - 5.16	105667	84704	1.247	1
30.16 - 18.16	363891	106818	3.407	0.00987 **
5.21 - 18.16	-210409	78145	-2.693	0.10636
18.21 - 18.16	2082	84585	0.025	1
30.21 - 18.16	100835	86214	1.17	1
5.21 - 30.16	-574300	102718	-5.591	3.39e-07 ***
18.21 - 30.16	-361809	107699	-3.359	0.01171 *
30.21 - 30.16	-263057	108983	-2.414	0.23684

18.21 - 5.21	212491	79344	2.678	0.11107
30.21 - 5.21	311244	81078	3.839	0.00185 **
30.21 - 18.21	98752	87303	1.131	1

Table A10-Results of multiple comparisons. examining the effects of salinity and temperature on D_{25Mg} in the otolith of *Solea senegalensis*. *p<0.05; **p <0.01; ***p<0.001

Fixed effects	Estimate	Std. Error	z value	Pr(>|z|)
18.16 - 5.16	-54968	131163	-0.419	1
30.16 - 5.16	333248	166336	2.003	0.6769
5.21 - 5.16 =	-234174	126798	-1.847	0.97159
18.21 - 5.16	28882	136870	0.211	1
30.21 - 5.16	149517	139468	1.072	1
30.16 - 18.16	388217	165815	2.341	0.28828
5.21 - 18.16	-179205	126114	-1.421	1
18.21 - 18.16	83850	136237	0.615	1
30.21 - 18.16	204485	138846	1.473	1
5.21 - 30.16	-567422	162384	-3.494	0.00713 **
18.21 - 30.16	-304367	170365	-1.787	1
30.21 - 30.16	-183732	172459	-1.065	1
18.21 - 5.21	263055	132039	1.992	0.69515
30.21 - 5.21	383690	134730	2.848	0.06603.
30.21 - 18.21	120635	144249	0.836	1