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Fanny Thibon[1*], Jean Goedert[2], Nicolas Séon[2], Lucas Weppe[1,3], Jeremy E. Martin[4], Romain Amiot[4], Sylvain Adnet[5], Olivier Lambert[6], Paco Bustamante[3,7], Christophe Lécuyer[4], Nathalie Vigier[1]

[1]Laboratoire d'Océanographie de Villefranche-sur-Mer (LOV), CNRS UMR 7093/Sorbonne Université; 06230 Villefranche-sur-Mer, France.

[2]Muséum National d'Histoire Naturelle (MNHN) - Centre de Recherche en Paléontologie – Paris (CR2P), CNRS UMR 7207/Sorbonne Université; 75231 Paris Cedex 05, France.

[3]Littoral Environnement et Sociétés (LIENSs), CNRS UMR 7266/La Rochelle Université; F-17000 La Rochelle, France.

[4]Laboratoire de Géologie de Lyon, Terre, Planètes et Environnement (LGL-TPE), Université Claude Bernard Lyon1/CNRS UMR 5276/École Normale Supérieure de Lyon; 69622 Villeurbanne Cedex, France.

[5]Institut des Sciences de l'Evolution de Montpellier (ISEM) – Université de Montpellier/CNRS UMR 5554/IRD/Ecole Pratique des Hautes Etudes; 34095 Montpellier Cedex 5, France.

[6]Direction Opérationnelle Terre et Histoire de la Vie, Institut Royal des Sciences Naturelles de Belgique; 1000 Brussels, Belgium.

[7]Institut Universitaire de France (IUF); 75005 Paris, France.

*Corresponding author: Fanny Thibon

Email: thibon.fanny@orange.fr

Abstract

The vertebrate fossil record documents a plethora of transitions between aquatic and terrestrial environments but their causes are still debated. Quantifying the salinity of living environments is therefore crucial for precising the sequence of ecological transitions. Here, we measured lithium stable isotope composition of mineralized tissues (δ^7Li_{mt}) of extant and extinct vertebrates from various aquatic environments: seawater, freshwater/terrestrial, and "transitional environments" (i.e. brackish waters, or seasonal access to freshwater and seawater). We report statistically higher δ^7Li_{mt} values for seawater vertebrates than freshwater ones, taxonomic groups considered separately. Moreover, vertebrates living in transitional environments have intermediate δ^7Li_{mt} values. Therefore, we show that δ^7Li_{mt} values of both extant and extinct vertebrates can discriminate their aquatic habitat.

Keywords: Lithium isotopes – Mineralized tissues – Aquatic environments – Paleoecology

1. Introduction

Vertebrate species can face variable water environments during their lifetime. As salinity varies drastically between marine waters and freshwater/terrestrial environments, it is a key parameter for vertebrates (Gutiérrez, 2014). However, these environmental shifts remain challenging and important to trace, notably to understand the cryptic life of modern aquatic vertebrate species, especially fish (Maxwell, 2007; Mesiar et al., 1990), or to shed light on the evolution of past aquatic vertebrate. Indeed, the aquatic vs terrestrial environmental dichotomy was pivotal during the evolution of life on Earth (Pyenson et al., 2014; Uhen, 2007), and vertebrates have adapted to salinity variations through complex morphological and physiological changes. It is therefore crucial to possess tools to characterize the aquatic habitat of current and past vertebrate species.

To this end, stable isotopes are commonly used as modern and past ecological proxies (Fry, 2006). They are notably applied to vertebrate mineralized tissues (bones, teeth, eggshells, scales, fish otoliths) to investigate current to past biological and physiological characteristics such as body temperature (Amiot et al., 2006; Séon et al., 2020), mobility (Fricke et al., 2009; Lazzerini et al., 2021; Lugli et al., 2019), trophic ecology (Hassler et al., 2018; Wißing et al., 2019), and to reconstruct living environments of ancient animals (Amiot et al., 2010; Goedert et al., 2020, 2018).

In recent decades, stable ($\delta^{13}C$, $\delta^{18}O$, $\delta^{34}S$) and radiogenic isotope systems ($^{87}Sr/^{86}Sr$) applied to vertebrate mineralized tissues have proved to be the best relevant palaeoecological proxies for reconstructing salinity and/or the environmental conditions of their aquatic environments (i.e. seawater vs freshwater) (Clementz et al., 2006; Fischer et al., 2012; Goedert et al., 2020, 2018; Kocsis et al., 2009; Schmitz et al., 1997). However, they also hold limitations. First, post-depositional diagenetic processes may affect the pristine isotopic composition of vertebrate mineralized tissues through element absorption or dissolution by contact between earth fluids and bioapatite. It is, however, worth noting that bone is more prone to extensive alteration than tooth enamel, which is resistant to dissolution-recrystallization processes as shown experimentally by Zazzo *et al.* (Zazzo et al., 2004). In parallel, some environmental estimations remain debated due

to other constraints of these isotopic proxies. Among them: (i) C and O are major elements essential to life and numerous biological processes can affect their isotopic distributions (Fry, 2006); (ii) $\delta^{18}O$ is a poor proxy of salinity at low latitude (Dansgaard, 1964); (iii) a weak marine influence can overshadow the continental signal recorded in bone $\delta^{34}S$ (Goedert et al., 2018); and (iv) $^{87}Sr/^{86}Sr$ values of terrestrial vertebrates can be mistaken as marine values depending on the type of bedrock inhabited (Holt et al., 2021); (v) significant overlap in $\delta^{13}C$ values may occur between marine vertebrates and terrestrial ones consuming C4 vegetation (e.g. Clementz et al., 2006). Furthermore, species migrating between waters of different salinities or living in brackish waters can have $\delta^{18}O$ or $\delta^{34}S$ signatures indistinguishable from species living in seawater (Goedert et al., 2020). Only the joint application of these two isotopic systems might overcome such potential bias, as two recent studies indicate (Goedert et al., 2020, 2018).

We address these challenges of assessing the aquatic environments of both modern and extinct vertebrate species by using a new stable isotopic proxy: lithium (Li). Unlike C and S, Li is not a nutrient. Lithium also displays a broader range of isotope fractionation than C and O (Fanny Thibon et al., 2021). Therefore, the Li stable isotope system presents a clear-cut advantage in distinguishing aquatic environments based on salinity differences. Earth's rocks reservoirs have $\delta^{7}Li$ values that vary among various environments (Penniston-Dorland et al., 2017), from -15 to +20 ‰. Dissolved Li concentration and isotope composition in freshwaters (lakes, groundwaters, rivers) display significant variations, from 0.1 to 22 ng/mL in concentration, and from +1 to +44 ‰ in $\delta^{7}Li$ values (Huh et al., 1998). In addition, due to its weak capacity to adsorb onto marine particles (Decarreau et al., 2012), seawater Li mainly exists under free Li^{+} ions forming stable complexes with four water molecules (Rempe et al., 2018; Rudolph et al., 1995). Since dissolved Li has a long oceanic residence time (~1.2 million years (Lécuyer, 2016)) far greater than the ocean mixing time, it is homogeneously distributed throughout the water column (Misra and Froelich, 2012) and between the oceans, which have both a constant Li concentration of 179 ng/mL (Broecker and Peng, 1982) and a $\delta^{7}Li$ value of +31.0 ± 0.5 ‰ (Millot et al., 2004). Based on recent palaeoceanographic reconstructions, seawater $\delta^{7}Li$ values remained constant during

the last 10 Ma, and progressively decreased to 22 ‰ 65 Ma ago (Misra and Froelich, 2012). Here, we report the first measured Li stable isotope compositions of vertebrate mineralized tissues (δ^7Li_{mt}; bones and teeth) from 74 modern and 11 fossil specimens inhabiting different types of environments (terrestrial, semi-aquatic, aquatic) and relying on either freshwater or seawater.

2. Materials and Methods

2.1. Sampling and sample preparation

74 modern and 11 fossil bones and teeth were sampled from 5 taxonomic groups, namely Chondrichthyes, Osteichthyes, Mammalia, Reptilia, and Amphibia.

2.1.1. Modern samples

Amphibian vertebrae (samples Pe-ri-1–5) were all collected from a breeding farm in Pierrelatte (Drôme, France). Freshwater fish vertebrae (samples Si-gl-1 and Cy-Ca-1) were collected from a lake in the Dombes region (Ain, France), operated by Maison Liatout. The majority of seawater fish vertebrae (samples Di-la-1, So-so-1, Li-li-1, Li li 2, Li-li-3, Ga-mo-1, Ga-mo-2, On-ne-1, Mu-su-1, Mu-su-2, Se-ma-1, Se-ma-2, Sp-au-1, Pl-pl-1, Di-la-2, Pa-pa-1, Sp-ca-1, Sa-sa-1, XG33 and TT3) were collected from fisheries and come from different locations specified in Table S1. The majority of Mammalia and Reptilia samples (bone powder collected from different parts of the skeleton, cf. Table S1, samples 50.001461, 50.001410, 50.001331, 50.001018, 50.001014, 50.001020, 50.001027, 50.001023, 50.002521, 50.000517, 50.002365, 50.001353, 50.001313, 50.000229, 50.001036, 50.001046, 50.000527, 50.001682, 50.001681, 50.002123, 50.002041, 50.002038, 50.002039, 50.000788) come from the collection of the Musée des Confluences (Lyon, France). Additional Mammalia vertebrae (CC6, DD3, MDD1, PV8) and tooth bulk (samples DD29, DD46, MDD36, PV42) were sampled from stranding specimens collected by PELAGIS and the Museum National d'Histoire Naturelle (MNHN, Paris, France). Two Mammalia bone fragment samples (JM18031 and JM1536) were collected from skeletons at the Institut Royal des

Sciences Naturelles de Belgique (IRSNB, Brussels, Belgium). The two samples of Mammalia dentine (JM1502B and Inia) were collected from the IRSNB and the Naturhistorisches Museum Basel (Switzerland) (sample N° 7167), respectively. The Chondrichthyes tooth bulk (REC0321M, REC0204M, REC0610M, REC0270M, REC 767, REC 592M, REC 706-711, REC 1570M and REC 1571M) are collection specimens from the Institut des Sciences de l'Évolution, Montpellier (ISEM), University of Montpellier, France.

2.1.2. Fossil samples

All the fossil samples (1-BAZ-19, 1-BAZ-23, 1-BAZ-8e, 1-BAZ-8d, 1-BAZ-17e, 1-BAZ-17d, 1-BAZ-4e, 1-BAZ-4d, 1-BAZ-14e, 1-BAZ-16e, 1-BAZ-16d) were collected from Miocene deposits that temporarily outcropped in the municipality of Bazas (Aquitaine Basin, France). More details are available in Goedert *et al.* (Goedert et al., 2017).

For all bone samples, we favored cortical bone. Bioapatite powder was collected either by crushing bone and tooth samples into a fine powder using an agate mill or by using a DremelTM equipped with a spherical diamond-tipped drill bit. Additional information (location, type of skeletal material) is given for each sample in Table S1.

2.1.3. Chemical preparation

All analytical preparations were performed in a pressure-cleaned laboratory under a fume-hood to minimize procedural blanks, using distilled reagents and pre-cleaned vessels. Approximately 5 to 10 mg of sample powder were first dissolved with a mixture of concentrated HNO_3 and H_2O_2. After 48 h, concentrated HCl was added to pursue the digestion in inverse aqua regia, and heated for 24 h before being evaporated.

Lithium extraction and purification were performed following a well-documented routine procedure (James and Palmer, 2000; Vigier et al., 2009), recently adapted to biological materials and tissues (Thibon et al., 2021). Samples were taken up in 0.5 mL of 1 N HCl and loaded on AG 50-X12 (200-400 mesh) resin in 8.5-cm-high Teflon columns. Lithium was eluted with 1 N HCl. A

second purification step, following the same procedure as the first Li extraction, ensured complete separation of Li.

Five total procedural blanks and five replicates of cow bone ash reference material (SRM-1400) were included. The precision and accuracy of the overall procedure were checked and published in Thibon *et al.* (Fanny Thibon et al., 2021).

2.2. Analytical measurements

Lithium has two naturally occurring isotopes (^{7}Li - ^{6}Li). The isotopic compositions of geological and biological materials are reported as per-mil variations relative to an isotopic standard (here NIST L-SVEC-1 SRM 8545) as $\delta^{7}Li_{sample}$ [‰] = $(((^{7}Li/^{6}Li)_{sample} / (^{7}Li/^{6}Li)_{LSVEC} - 1) \times 1{,}000$. Li isotopic analyses were performed using a Thermo-Fisher Neptune Plus multi-collector inductively coupled plasma mass spectrometer (LGL-TPE UMR CNRS 5276, France) following the procedure developed for low Li concentrations in biological materials (Balter and Vigier, 2014; Bastian et al., 2018; Fanny Thibon et al., 2021). Samples and standards were analyzed under dry plasma conditions in low-resolution mode. The standard bracketing method (using the LSVEC reference material) was used to performed instrumental mass bias correction. The five total Li procedural blanks, which included chemical and analytical blanks, ranged between 20 and 40 pg. For 10 mg of material with a Li concentration of 1 µg/g, the maximum blank contribution was negligible (0.4 %). On average, the internal reproducibility was ± 0.04 ‰. Precision (external reproducibility) was estimated from 2 to 6 replicate measurements of 29 different samples, and ranged from 0.1 to 0.9 ‰ (2SD). Cow bone ash reference material (SRM-1400), previously published at -1.7 ± 0.1 ‰ (2SE) (Fanny Thibon et al., 2021), was measured here at -1.9 ± 0.3 ‰ (2SE, n = 5), which confirmed the procedure's accuracy. Li concentrations have been estimated using voltage measurements during isotopic measurements (using a normalization to a LSVEC calibrated solution).

2.3. Body mass estimation

We additionally reported the body mass for Chondrichthyes and Osteichthyes species (Tables S1). When the body mass was not initially measured, but the total length was, we used length-weight relationships: (LWR) weight = a(total length)exp(b) (Froese et al., 2014), with the LWR parameters a and b taken from www.fishbase.org.

2.4. Statistical treatments

Since normality and homoscedasticity of the Li isotopic data were not validated, the non-parametric Mann-Whitney-Wilcoxon and Kruskal-Wallis tests were used to compare median values between two or more observational series, respectively. Tests were performed using R software (R Core Team, 2017) using the functions *wilcox.test* or *kruskal.test*. The level of significance for statistical analyses was set at p-val < 0.05.

A linear model was used to decipher which factors play a key role in the Li isotope compositions. The predictors applied to the model are the body mass, trophic levels, Li concentrations, taxonomic group, water mode (seawater/freshwater/intermediate water), and life mode (aquatic/semi-aquatic/terrestrial).

3. Results and Discussion

According to the linear model, only 3 variables are significant predictors: water and life modes (p-val < 0.001), and taxonomic group (p-val < 0.05). The following sections, therefore, focus on these variables.

3.1. Characterizing aquatic environments using δ^7Li_{mt}

δ^7Li_{mt} values of modern aquatic and terrestrial vertebrates cover an extensive range, from -24.0 to +36.0 ‰. While their Li concentrations are relatively low, they also vary considerably, from 0.1 to 6.3 µg/g dry weight (Fig. S1 and Table S1). Considering taxonomic groups separately, vertebrates living in seawater have, on average, significantly higher δ^7Li_{mt} values (26.8 ± 6.8 ‰ (SD, n=7), 16.5 ± 3.4 ‰ (SD, n=13), 9.0 ± 5.6 ‰ (SD, n=20) for Chondrichthyes, Osteichthyes

193 and Mammalia, respectively) than those in freshwater (-2.8 ± 3.0 ‰ (SD, n=2), -5.0 ± 4.6 ‰ (SD,
194 n=2), -9.0 ± 7.4 ‰ (SD, n=9) for Chondrichthyes, Osteichthyes and Mammalia, respectively;
195 Wilcox. test, *p*-val < 0.05, < 0.05, and < 0.0001, respectively; Figs. 1 and 2, Table 1). However, Li
196 concentrations of mineralized tissues are not significantly different between vertebrates living in
197 seawater and freshwater (1.0 ± 0.6 µg/g (SD, n=20) vs. 0.5 ± 0.3 µg/g (SD, n=9) for Mammalia
198 living in seawater and freshwater; Wilcox. test, *p*-val > 0.05), except for Chondrichthyes and
199 Osteichthyes (2.0 ± 0.7 µg/g (SD, n=7) and 2.0 ± 0.9 µg/g (SD, n=13), for Chondrichthyes and
200 Osteichthyes living in seawater vs 0.4 ± 0.2 µg/g (SD, n=2) and 0.4 ± 0.2 µg/g (SD, n=2), and for
201 Chondrichthyes and Osteichthyes living in freshwater; Wilcox. test, *p*-val < 0.05, Figs. S1 and
202 S2).

203 The Li_{mt} concentrations and δ^7Li_{mt} values of bones from five marsh frogs (*Pelophylax ridibunda*),
204 raised in the same basin and sharing the same water and food resources, are homogenous and
205 vary from 0.36 µg/g to 0.53 µg/g with a mean of 0.46 ± 0.06 µg/g (SD, n = 5), and from -12.9 ‰ to
206 -7.6 ‰ with a mean of -9.6 ‰ ± 2.1 ‰ (SD, n = 5), respectively. The same comparisons cannot
207 be made with teeth since teeth from different individuals of the same species living in similar
208 environments were not sampled in this study. As for bone and tooth samples from single
209 individuals, distinct Li_{mt} concentrations and δ^7Li_{mt} values were recorded, showing tooth-bone
210 differences ranging from 0.2 µg/g, for the harbour seal (*Phoca vitulina*) to 1.7 µg/g for the short-
211 beaked common dolphin (*Delphinus delphis*), and from -2.5 ‰ for the short-beaked common
212 dolphin to +10.1 ‰ for the harbour seal (Table S2). Nevertheless, these differences fell within
213 value ranges corresponding to the same aquatic environmental condition. Both inter- and intra-
214 individual δ^7Li_{mt} value variabilities potentially result from isotopic fractionations during biological
215 and physiological processes. As reported by Poet, Vigier, Bouret et al. (submitted to iScience), Li
216 isotopes are strongly fractionated by cell transporters, and therefore may lead to variable isotopic
217 compositions between organs (tooth vs. bones) and between individuals from the same species.
218 These isotopic effects were also detected in mussels cultured under various conditions (Thibon et
219 al., 2021). Nevertheless, these variabilities do not overprint the environmental variability. Indeed,

δ^7Li_{mt} values can distinguish between Osteichthyes living in seawater, freshwater, and "transitional environments", i.e. brackish waters, or seasonally crossing waters of different salinities, such as the sockeye salmon (*Onchorynchus nerka*) (Figs. 1 and 2, δ^7Li_{mt} values being +16.5 ± 3.4 ‰ (SD, n=13), +9.9 ± 2.9 ‰ (SD, n=8), and -5.0 ± 4.6 ‰ (SD, n=2), respectively for fish living in seawater, transitional environments, and freshwater). δ^7Li_{mt} values of fish species living in transitional environments are intermediate and statistically different from δ^7Li_{mt} values of fish living in seawater and freshwater (Wilcox. test, *p*-val < 0.05 and < 0.0001, respectively, Fig. 2). Furthermore, the Li concentrations of their mineralized tissues (Li_{mt}) also reflect their living environment since Osteichthyes Li_{mt} values are statistically different between seawater, freshwater, and transitional environments (respectively 2.0 ± 0.9 µg/g (SD, n=13), 0.4 ± 0.2 µg/g (SD, n=2), 1.4 ± 0.4 µg/g (SD, n=8), Wilcox. test, *p*-val < 0.05, Fig. S2). Consequently, both δ^7Li_{mt} values and Li concentrations allow clear discrimination between seawater/transitional/freshwater groups (Fig. S6). Water salinity thus has a noticeable effect on Li accumulation, transport, and/or excretion in vertebrate mineralized tissues. In contrast, O and S isotopes need to be considered jointly to distinguish fish species living in transitional environments (Goedert et al., 2020, 2018) (Figs. 3a and 3b). The Li entry in biological cells can explain its isotope variability between environments of different salinity. Indeed, the two major ions that influence salinity are chloride and sodium, although salinity is based on a large diversity of elements. Lithium can enter biological cells through Na/H exchangers (NHE) following Na ions (Counillon et al., 2016). The competition between Li and Na leads to Li isotope fractionation that varies according to water Li/Na ratio, up to ~13 ‰ in favor of the light isotope ^{6}Li (Poet et al., submitted to iScience). Li bioaccumulation may also be different in Li-rich (seawater) and Li-poor (river waters) environments, and this may also affect Li isotope composition of biological tissues (Thibon et al., 2021).

The first straightforward application of Li stable isotope compositions in vertebrate mineralized tissues concerns modern ecology. Numerous fish species spend time in both marine and freshwater environments throughout their lifetime (Jones, 2006; Quinn, 2018). Furthermore, many

247 species inhabiting transitional environments, such as estuaries, are spatially distributed along a
248 salinity gradient (Jones, 2006). These habitats are difficult to establish solely from visual
249 observation of collected specimens. Indeed, routine monitoring techniques, such as acoustic or
250 satellite telemetry through physical tracking (Hussey et al., 2015), or hydroacoustic surveys
251 (Maxwell, 2007; Mesiar et al., 1990), encounter obstacles when following species that are highly
252 mobile, undertake large-scale migrations, or have high migrant numbers. Due to the broad
253 variability of $\delta^{7}Li_{mt}$ values in the environment and the clear distinction between marine and
254 freshwater/terrestrial environments, Li stable isotopes are useful for elucidating living
255 environments. In addition, stable isotope compositions such as $\delta^{13}C$, $\delta^{15}N$ or $\delta^{34}S$, while being
256 powerful tools for identifying and characterizing environmental niches of fish species (Dwyer et
257 al., 2020; Fry and Chumchal, 2011) commonly rely on soft tissue such as muscles. Li stable
258 isotopes therefore offer an additional approach applicable to solid mineralized tissues of modern
259 fish, with $\delta^{7}Li_{mt}$ of paramount interest in transitional environments.

260

261 3.2. Biological imprints on the $\delta^{7}Li_{mt}$ record

262 Although $\delta^{7}Li_{mt}$ values reflect the living environments of modern species, they also display
263 important variations within a single environment. These variation ranges are of 19.7 ‰ (n = 7),
264 10.7 ‰ (n = 13), 22.0 ‰ (n = 20), and 10.1 ‰ (n = 3) for modern seawater Chondrichthyes,
265 Osteichthyes, Mammalia, and Reptilia, respectively, and 4.3 ‰ (n = 2), 6.5 ‰ (n = 2), 22.3 ‰ (n
266 = 9) and 6.3 ‰ (n = 4) for modern freshwater counterparts. Yet, these variations do not mask the
267 influence of the environment, especially for Chondrichthyes and Osteichthyes, since no overlaps
268 in $\delta^{7}Li_{mt}$ values are reported in the present dataset (Figs. 1 and 2). We thus investigated
269 biological parameters that could affect $\delta^{7}Li_{mt}$ values from the same taxonomic group within similar
270 environments and also explain differences in values between two taxonomic groups inhabiting
271 similar environments.

First of all, as lithium is effectively incorporated in marine species by filtration and by predation (Thibon et al., 2020), the quantity of filtrated water may impact both Li concentration and isotopic composition of Chondrichthyes and Osteichthyes biological mineralized tissues. The amount of filtrated water directly depends on the gill surface area, which is linked to the body mass (Bigman et al., 2018). A positive relationship between δ^7Li_{mt} values and body mass is highlighted in Fig. 4 ($r = 0.69$, p-val < 0.001). In this way, a metabolic effect may affect δ^7Li_{mt} values. As mentioned above, Li can enter biological cells through NHE (Counillon et al., 2016). The latter can fractionate Li isotopes in favor of the light isotope ^6Li (Poet et al., submitted to iScience. As for isotopic fractionation occurring during Rayleigh distillation, it seems that the more water that is filtrated, the more Li passing through gill cell NHE, but the less Li isotopically fractionated with respect to seawater. This could explain why the heavier Chondrichthyes displays δ^7Li_{mt} values close to seawater's, and the smaller Osteichthyes, much lower values (Fig. 4). Furthermore, this can also explain the proximity of δ^7Li_{mt} values between Osteichthyes and Chondrichthyes of close body mass (Figs. 1 and 4). However, this hypothesis must be further investigated due to differences between Osteichthyes and Chondrichthyes gill anatomy and physiology (Evans et al., 2005).

On average, cetaceans living in seawater have δ^7Li_{mt} values lower than marine Osteichthyes or Chondrichthyes (respectively +12.3 ± 4.4 ‰ (SD, n=10), +16.5 ± 3.4 ‰ (SD, n=13), +26.8 ± 6.8 ‰ (SD, n=7), Fig. 1, p-val < 0.05). Significantly, their principal Li source, differently from Osteichthyes and Chondrichthyes, is food ingestion. This suggests that, secondarily to filtration, supplementary biological mechanisms fractionate Li stable isotopes during intestinal assimilation, ultimately favoring the relative incorporation of ^6Li in bones.

Meanwhile, terrestrial vertebrates such as mammals have lower δ^7Li_{mt} values than semi-aquatic or aquatic freshwater mammals, except for the Amazon river dolphin (*Inia geoffrensis*). This is also the case for terrestrial and semi-aquatic birds originating from the same geographic area (Fig. 1). This tendency hints at potential fractionation mechanisms in terrestrial environments resulting in relative concentration of the lighter isotope (^6Li) in mineralized tissues.

299 It is worth noting that δ^7Li_{mt} values are also valuable for tracking the feeding habits of vertebrates.
300 For instance, the polar bear (*Ursus maritimus*) and the sea otter (*Enhydra lutris*) have δ^7Li_{mt}
301 values of +11.3 ‰ and + 8.03 ‰, respectively, which fall within the range of seawater aquatic
302 mammal values. Although terrestrial species rely on freshwater for their water supply, polar bears
303 and sea otters feed on marine aquatic species such as seals or molluscs, hence their δ^7Li_{mt}
304 values. In modern ecology, δ^7Li_{mt} values thus represent a reliable tool to test marine resource
305 uptake in terrestrial species (Bicknell et al., 2020).

306

307 3.3. Palaeoecological implications

308 Overall, δ^7Li_{mt} values of Miocene fossil vertebrates from the Aquitaine Basin (France) are
309 consistent with observed modern δ^7Li_{mt} values and clearly discriminate between marine and
310 freshwater vertebrate taxa (Fig. 1). All fossil δ^7Li_{mt} values fall within the seawater/freshwater
311 ranges defined by the present-day samples, apart for the dentine sample of the terrestrial
312 mammal *Gomphotherium angustidens*, and agree with stable oxygen isotope compositions from
313 a previous study (Goedert et al., 2017). Of particular note, the highest fish δ^7Li_{mt} value,
314 corresponding to the fossil fish *Trigonodon jugleri*, falls within the range of modern seawater fish.
315 This may be linked to the specimen's size. Indeed, even though its modern counterparts
316 (*Pseudodax moluccanus*) measure approximately 25 cm, Miocene *T. jugleri* tooth size indicates a
317 body length of about 1 meter, assuming isometry (Schultz and Bellwood, 2004), making it
318 comparable to the largest modern marine fish we sampled, namely the Bluefin tuna (*Thunnus*
319 *thynnus*). As discussed in previous sections, filtration through the gills may explain this extreme
320 value, and supports a larger specimen size as suggested by Schultz & Bellwood (Schultz and
321 Bellwood, 2004).

322 A body size effect on Li isotope compositions is however ruled out for the fossil Chondrichthyes
323 *Otodus megalodon* and *Cosmopolitodus hastalis* — large species with slightly lower δ^7Li_{mt} values
324 than their modern seawater counterpart. *O. megalodon* spatial distribution was wide: nurseries

have been found in shallow waters while adults lived in the open ocean far from coasts (Pimiento et al., 2010). Furthermore, these sharks inhabited waters with annual mean temperatures from 12 to 27 °C (Pimiento et al., 2016). Indeed, they could efficiently regulate their body temperature, allowing them to dwell in cold waters (Pimiento et al., 2016). In addition, they may have frequented in deep waters like their modern analogue, the great white shark (Pimiento et al., 2016), which can dive as deep as 1000 m (Skomal et al., 2017). Therefore, their habitat may have had different salinity ranges than modern sharks, and additional biological factors could have influenced their Li isotopic signatures.

As for sirenians, both seawater subfossil Steller's sea cow (*Hydrodamalis gigas*) and modern dugong (*Dugong dugon*) samples display δ^7Li values close to those of the fossil Dugongidae (+3.7, +7.7, +6.7 vs +5.2 ‰, respectively, see Fig. 1 and Table S2). This supports the notion that after mammals recolonized aquatic environments in the Eocene (cetaceans and sirenians), some species (Miocene Dugongidae) stayed in seawater environments while others, such as some Trichechinae, recolonized freshwater environments (Suarez et al., 2021).

In contrast to traditional isotopic systems (C, O, S, Sr), there is little insight on the behavior of Li in apatite during fossilization processes. Important shifts can be expected if recrystallization of secondary minerals occurs in a diagenetic fluid impacted, for example, by clay dissolution (rich in Li, and with low δ^7Li compared to waters). Nevertheless, in fossil carbonates (i.e., foraminifera), it has already been shown that certain early diagenetic histories have a negligible influence on Li isotopes (Misra and Froelich, 2012). We can therefore expect good signal preservation in apatites, a mineral that is thermodynamically more stable than carbonates. Here, we observe no clear relationship between fossil δ^7Li_{mt} values and Li concentrations (p-val > 0.05, Fig. S3), suggesting a negligible effect of diagenesis on Li and δ^7Li. Furthermore, no significant correlation is observed between δ^7Li_{mt} values or Li_{mt} and elements classically used as diagenesis proxies, such as Rare Earth Elements, data previously published in Goedert *et al.* (Goedert et al., 2017) (R^2 = 0.04 and 0.19, respectively; p-val > 0.05; Fig. S4). Nevertheless, fossil dentine and enamel from *O. megalodon* and Rhinocerotidae have similar δ^7Li_{mt} values (+14.8 vs +14.6 ‰ and -1.4 vs

-1.7 ‰, respectively, Fig. S5 and Table S2). This, however, is not the case of *C. hastalis* and *G. angustidens* (+13.9 vs +21.9 ‰ and +8.0 vs -5.0 ‰, respectively, Fig. S5 and Table S2). Being more porous than enamel, dentine is more prone to chemical alterations during diagenesis. Indeed, Li is highly mobile in aqueous fluids, and may be lost from dentine during diagenesis, altering the pristine Li isotope composition. However, almost all fossil vertebrate samples have higher Li concentrations than their present-day counterparts (Fig. S1 and Table 1). Therefore, either a Li gain took place during diagenesis without deeply affecting the isotopic composition, or environmental Li concentrations were higher in the past or at least in this specific paleoenvironmental context. It should be noted that systematic Li gain during diagenesis is not expected due to Li incompatibility with apatite (Brophy et al., 2011; Penniston-Dorland et al., 2017). The second explanation is therefore more likely, considering that oceanic Li concentration changed in the past (Misra and Froelich, 2012), and was potentially higher during the Burdigalian (Hathorne and James, 2006). In addition, a higher oceanic Li concentration could have been triggered by a higher Li river flux (Hathorne and James, 2006), thus also explaining the higher Li environmental level for terrestrial fossils. All these hypotheses need further testing by additional studies on both fossil and modern samples. Nevertheless, fossil data strongly support the hypothesis that extinct vertebrate $\delta^{7}Li_{mt}$ values directly reflect the seawater/freshwater dichotomy as present-day $\delta^{7}Li_{mt}$ values do.

Finally, in the field of archaeology, many studies investigate the foods items consumed by human populations (Ambrose and DeNiro, 1986). One recurrent question concerning the consumption of marine vs terrestrial resources, especially among populations living near coastal areas, is currently addressed by stable isotope compositions such as $\delta^{15}N$ or $\delta^{34}S$ (Lécuyer et al., 2021). As discussed in section 3.2, $\delta^{7}Li_{mt}$ values may also be valuable for tracking the feeding habits of vertebrates. Here again, Li has the potential to become powerful for detecting the consumption of marine resources by terrestrial vertebrates.

Another advantage Li isotopes in mineralized tissues bring to paleo-ecological questions is that soft tissues from deep times (> 1 Ma) have disappeared, leaving only mineralized tissues in

sediments, namely an abundance of vertebrate bones, teeth, or fish scales in the fossil record. According to this preliminary study, at least part of the pristine Li isotopic signature appears to be preserved in enamel. Being more sensitive to transitional environments than oxygen or sulfur isotopes, Li isotopes in fossil enamel of Devonian tetrapods, for example, may provide new clues about their terrestrialization.

4. Conclusions

These results demonstrate that Li stable isotope compositions in bones and teeth from extant vertebrate species constitute a valuable proxy for determining their aquatic environments, i.e. seawater, freshwater, or transitional. Additional second-order variability might reflect biological imprints such as the amount of water filtrated through fish gills. Moreover, Li isotopes in teeth and bones might also benefit to the reconstruction of past environmental conditions using fossil specimens. Although preliminary, the application of Li isotopes to fossil specimens already underlines their potential, either for establishing biological features such as size/body mass in relation to the quantity of filtrated water, as exemplified by *T. jugleri*, the habitat of *O. megalodon* and *C. hastalis*, or the environmental conditions of aquatic habitat like for the fossil *Dugongidae*. Overall, Li isotopes in mineralized tissues have the makings of a powerful tool for tracking the ecology of past and present-day vertebrates. This new isotopic tool might benefit the fields of ecology, archaeology and palaeontology by, respectively, tracking the living environments of vertebrate species, determining whether past human populations relied on marine resources, and resolving major past ecological transitions

Acknowledgments

Funding: Agence Nationale de la Recherche (ANR). Grant number: ANR-18-CE34-0002. Project title: ISO2MET (www.iso2met-project.fr). We are grateful for access to facilities provided by the Laboratoire Océanographique de Villefranche-sur-mer (LOV) and Laboratoire de Géologie de

Lyon, Terre-Planète-Environnement (LGL-TPE). JEM thanks L. Costeur (NHM Basel) for permitting the sampling of *I. geoffrensis*. NS thanks F. Demaret (PELAGIS, UMS3462), W. Dabin (PELAGIS, UMS3462) and C. Lefèvre (MNHN Paris) for permitting the sampling of bone and tooth on several marine mammals. OL thanks O. Pauwels and S. Bruaux (IRSNB) for permitting the sampling of bone and tooth on several marine mammals. FT and NV thank M. Montanes for her management of the clean lab at LOV and F. Arnaud-Godet and P. Telouk for their help with MC-ICP-MS at ENS Lyon. JG thanks the Musée des Confluences de Lyon and the curator M. Berthet for providing access to skeletal material and for permission to sample specimens. The IUF (Institut Universitaire de France) is acknowledged for its support to PB as a senior member. We thank the editor and the two anonymous reviewers who helped improving this manuscript.

Competing Interest Statement: The authors declare no competing interests

Data Availability: All data underlying the study are in supplementary Data files (.xlsx format).

Author Contributions: CRediT author statement. F. **Thibon**: Conceptualization, Supervision, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. J. **Goedert**: Conceptualization, Resources, Investigation, Writing - review & editing. N. **Séon**: Resources, Investigation, Writing - review & editing. L. **Weppe**: Methodology, Validation, Investigation, Writing – review & editing. J.E. **Martin**: Conceptualization, Resources, Investigation, Writing - review & editing. R. **Amiot**: Resources, Investigation, Writing - review & editing. S. **Adnet**: Resources, Investigation, Writing - review & editing. O. **Lambert**: Resources, Investigation, Writing - review & editing. P. **Bustamante**: Funding acquisition, Writing - review & editing. C. **Lécuyer**: Conceptualization, Investigation, Writing – review & editing. N. **Vigier**: Resources, Funding acquisition, Investigation, Writing - review & editing.

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593 **Figures and Tables**

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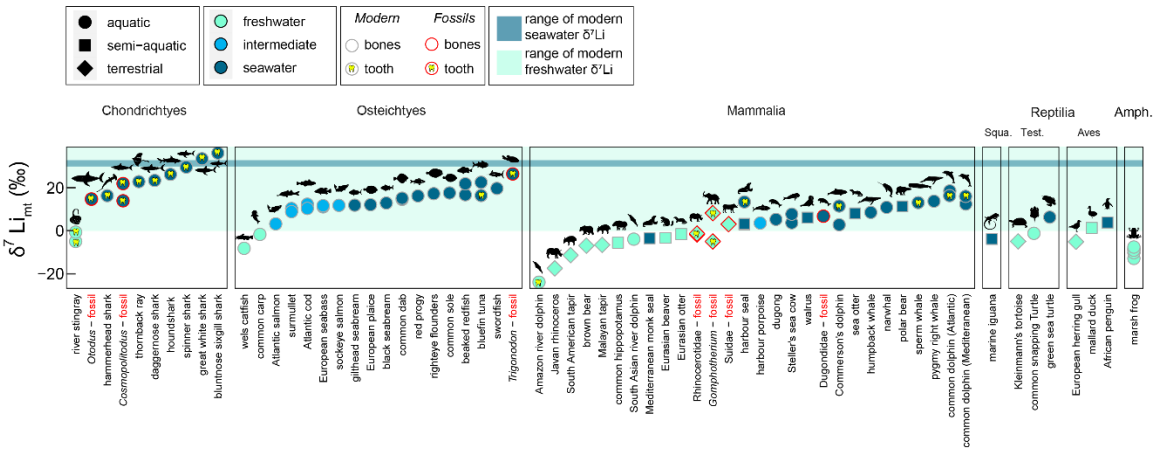


596 **Figure 1. δ^7Li_{mt} values of modern and fossil vertebrates including Chondrichthyes,**
597 **Osteichthyes, Mammalia, Reptilia, and Amphibia.** Each icon represents an independent
598 individual, except when tooth and bone from the same individual were sampled. Squa. for
599 Squamata, Test. for Testudinea, Amph. for Amphibia. Vertebrates living in seawater have, on
600 average, higher δ^7Li_{mt} values than those living in intermediate environments, which in turn display
601 higher δ^7Li_{mt} values than vertebrates depending on freshwater. SD are included in the size of the
602 markers.

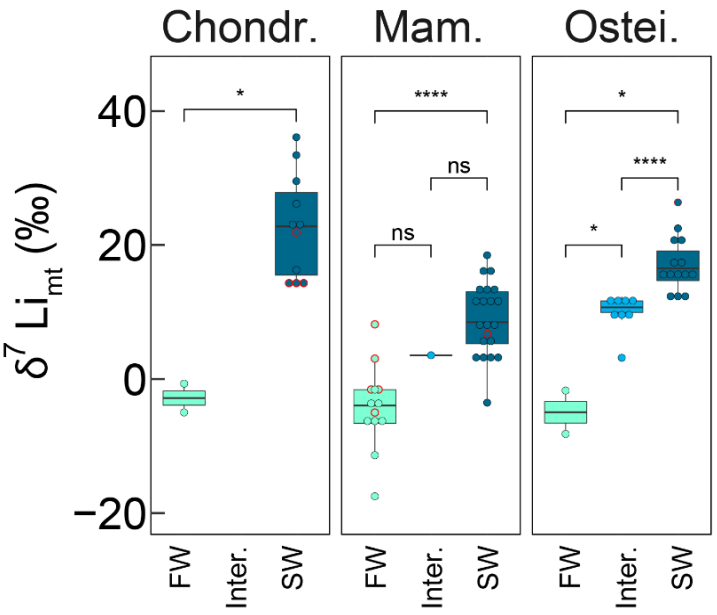
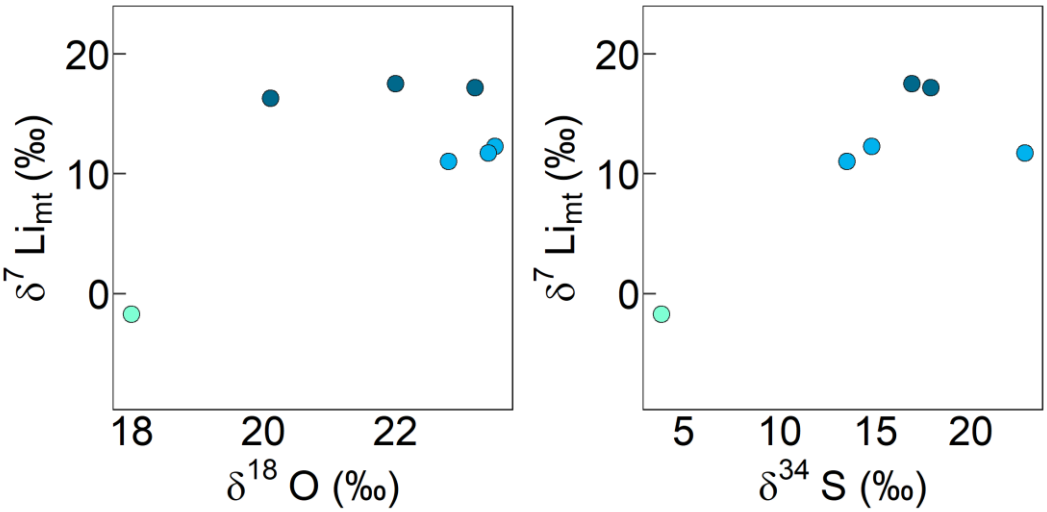
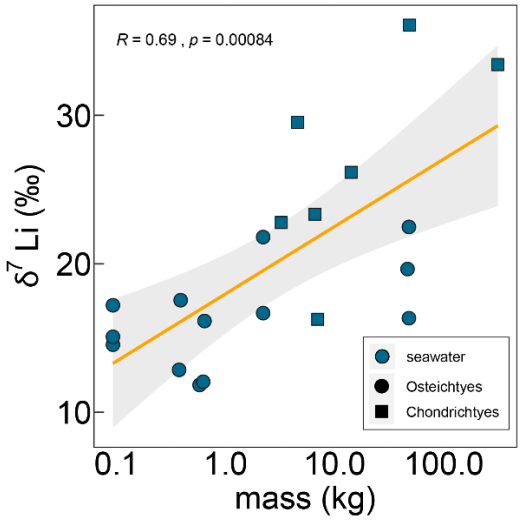


Figure 2. Distinction of water environments based on δ^7Li_{mt} values. Boxplot showing Chondrichthyes, Mammalia, and Osteichthyes δ^7Li_{mt} values according to their habitat (FW for freshwater, Inter. for intermediate water, and SW for seawater). Individual icons are added on top of each boxplot. Circles with red contours are fossil samples. Asterisks indicate *p*-values from the Mann-Whitney-Wilcoxon test (see Method section for details) and highlight the significance of the observed differences between pairs of groups: ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, **** for $p < 0.0001$. Chondrichthyes and Mammalia living in seawater have statistically higher δ^7Li_{mt} values than those living in freshwater environments. For Osteichthyes, species depending on intermediate environments have intermediate – and statistically different – δ^7Li_{mt} values between the ones for seawater and freshwater species.



614

615 **Figure 3. Modern Osteichthyes δ^7Li_{mt} values** compared to their corresponding (A) $\delta^{18}O$ values
616 and (B) $\delta^{34}S$ values. $\delta^{34}S$ and $\delta^{18}O$ values are from Goedert et al. (Goedert et al., 2020). See Fig.
617 1 for color legend. In contrast with $\delta^{34}S$ and $\delta^{18}O$ values, δ^7Li_{mt} values alone can discriminate
618 between fish living in seawater and intermediate environments.



619

620 **Figure 4. δ^7Li_{mt} values vs individual body mass for modern Chondrichthyes and**
621 **Osteichthyes**. The orange line is the linear regression line (regression coefficient r^2=0.47), and
622 the surrounding grey area is the 95 % confidence interval. R is the Pearson correlation
623 coefficient. A strong and positive correlation exists between δ^7Li_{mt} values and individual body
624 mass.

				δ^7Li_{mt} (‰)				Li_{mt} (µg/g)			
			n	min	max	mean	SD	min	max	mean	SD
Chondrichthyes	seawater	modern	7	16.3	36.0	26.8	6.8	1.1	3.1	2.0	0.7
		fossil	4	13.9	21.9	16.3	3.7	3.0	4.9	4.1	0.9
	freshwater	modern	2	-5.0	-0.7	-2.8	3.1	0.2	0.5	0.4	0.3
Osteichthyes	seawater	modern	13	11.8	22.5	16.5	1.3	0.7	4.0	2.0	0.9
		fossil	1			26.4				6.9	
	freshwater	modern	2	-8.2	-1.7	-5.0	4.6	0.2	0.6	0.4	0.2
Mammalia	seawater	modern	20	-3.5	18.5	9.1	5.6	0.2	2.3	1.0	0.5
		fossil	1			6.7				1.8	
	freshwater	modern	9	-23.9	-1.5	-8.9	7.4	0.1	1.2	0.6	0.3
		fossil	5	-5.0	8.1	0.6	5.1	2.5	19.0	7.8	6.6
Reptilia	seawater	modern	3	-3.8	6.3	2.1	5.3	0.7	1.2	0.9	0.3
	freshwater	modern	4	-5.1	1.3	-2.5	3.1	0.5	2.2	1.0	0.8
Amphibia	freshwater	modern	5	-12.8	-7.6	-9.6	2.1	0.4	0.5	0.5	0.1

Table 1. δ^7Li_{mt} values and Li_{mt} concentrations of modern and fossil vertebrates including Chondrichthyes, Osteichthyes, Mammalia, Reptilia, and Amphibia. "n" represents the number of samples analysed, "min" the minimum value, "max" the maximum value, "mean" the average value, and "SD" the standard deviation.