Title:

Phenotypic diversity of brown trout (*Salmo trutta* L.) during the Pleistocene-Holocene transition in the Iberian glacial refugium

Keywords: brown trout, phenotypic variation, Upper Palaeolithic, ZooMS, Stable Isotope analysis, sclerochronology

Abstract:

Human populations relied on fish for food for thousands of years, as evidenced in numerous Palaeolithic archaeological sites across Europe. A better understanding of past aquatic ecosystems provides insights into fish ecology and evolution, the impact of past climate changes on intraspecific biodiversity, and ultimately, human reliance on fish resources in the past.

In this study, we investigated past populations of brown trout (*Salmo trutta L.*) along the Bay of Biscay coast before, during, and after the Last Glacial Maximum (LGM), 33–9 kyr B.P. and compared their phenotypic diversity with modern populations. A total of 270 salmonid vertebrae from 11 archaeological sites in Spain and France were analysed. Species identification, combining ZooMS analyses with morphological traits and vertebral body size, confirmed 218 specimens as brown trout. A model for Salmonids species identification based on vertebral size is presented. Stable isotope analyses of carbon and nitrogen revealed a continuum of ecotypes. After testing multiple published regression models for body length estimation in brown trout, we developed a novel linear regression model alongside a protocol for age estimation. Overall, the analysis of body size, age and migratory ecotype demonstrated that the phenotypic diversity of brown trout populations during the Pleistocene-Holocene transition is comparable to that of modern populations, reinforcing the role of the Iberian glacial refugium in maintaining diversity during the LGM. The presence of large freshwater fish in human-occupied archaeological sites further suggests the exploitation of both riverine and marine fish resources.

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1. Introduction

The climatic shifts of the Quaternary period had a strong impact on species evolution and present ecology (Hewitt, 2000). In response to climate changes, most species relocated to more temperate, ice-free southern regions (i.e., glacial refugia, [Hewitt 2000; Kettle et al. 2011]), from which they later recolonized abandoned areas as conditions improved. The study of intraspecific variation during a glaciation-deglaciation transition provides insight into species resilience, adaptative patterns, and current distributions (Cortés-Guzmán et al., 2024; Hewitt, 2000), offering crucial information for predicting future trends under ongoing climate change (Dawson et al., 2011; Fordham et al., 2020). The last glacial maximum (LGM), occurring during the Late Pleistocene (26-10 kyr B.P.), is greatly studied, with numerous works focusing on its impact on species distribution and ecology in terrestrial organisms (e.g., Jones et al., 2020, 2021; Magri et al., 2006; Meiri et al., 2013).

During the LGM, Spain, Portugal and southern France remained ice-free except for limited areas of mountain glaciation, making the Iberian Peninsula also an important glacial refugium for fish (Kettle et al., 2011). Despite the important role of aquatic resources in human subsistence and their sensitivity to climatic changes, the impact of the LGM on aquatic ecosystems is still understudied. To this end, brown trout (*Salmo trutta* Linnaeus 1758) might be a relevant model due to its high phenotypic diversity, adaptation capacity, and recurrence in Palaeolithic archaeological sites. In particular, the Iberian Peninsula was indeed a refugium for salmonids, with numerous remains retrieved in archaeological sites in northern Spain and France (Adán et al., 2009; Barbaza & Heinz, 1992).

Brown trout is a diadromous fish, reproducing in the headwater stretches of rivers. The migration of this species to marine habitats (anadromous ecotype) is facultative. Brown trout populations including varying proportions of anadromous and resident fish are observed along the Atlantic coast. Brown trout alternative phenotypes result from a continuum of variation in migration patterns and phenology. This includes variations in the temporal and spatial amplitudes of migration to the sea, and differences in age at migration, along with variations in diet (Etheridge et al., 2008; Ferguson et al., 2019). The migratory status and diet types result in a wide range of growth rates, size at maturation and fertility, with body size and growth rates of migratory brown trout being much higher than their resident counterparts (Ferguson, 2006). The partition into alternative life strategies, anadromous or resident, is growth-dependent and controlled by genetic and environmental factors (Acolas et al., 2012; Ferguson et al., 2019).

The growth rate of brown trout depends greatly on temperature, which thus modulates also age at migration (Jonsson & L'Abée-Lund, 1993). During the ice sheet maximum extension between 26 and 19 kyr B.P. (Clark et al., 2009), lower temperatures might have determined reduced growth rates in brown trout and, in general, lower productivity in the ecosystem. This effect was possibly higher in freshwater environments, wherein temperatures are less buffered than at sea (Jonsson & L'Abée-Lund, 1993; LeRoy Poff et al., 2002), making the migratory strategy more advantageous in terms of body size, growth rate, and thus fertility, at least in females. Beginning around 19 kyr B.P., with glacial ice melting, large freshwater fluxes entered surrounding oceans, gradually increasing sea levels and enhancing both freshwater flow and nutrient input (Clark et al., 2009). These improved conditions might have determined an increase in growth rates and productivity in freshwater and marine environments, resulting in higher population densities that triggered the exploration of new environments. Dispersal of brown trout is achieved with anadromy (Elliott, 1994). Therefore, the presence of migratory populations with large body sizes might have been crucial for post-glacial re-colonisation (Ferguson, 2006). Moreover, anadromous fish were possibly preferentially targeted by fishing activity due to their larger body size and seasonal migration, and are possibly more represented in ichthyo-archaeological assemblages (e.g., Halffman et al. 2015).

Most studies on Iberian archaeological salmonids focused on taxonomic identifications (Roselló-Izquierdo et al., 2016, 2024; Roselló-Izquierdo & Morales-Muñiz, 2014). Likewise, little is known about the ecology of these fish during the LGM (Morales Muñiz & Roselló-Izquierdo, 2016; Morales-Muñiz & Roselló-Izquierdo, 2008; Roselló-Izquierdo et al., 2024). In Asturias, no major differences in size and growth between Palaeolithic and modern salmonids (Turrero et al., 2012) were found, but the lack of discrimination between Atlantic salmon (*Salmo salar* Linnaeus 1758) and brown trout, which have different modern ecologies, weakens the conclusions of this study which is a problem as underlined in Morales Muñiz et al. (2021). In a study on the ichthyo-archaeological assemblage of Middle-Upper Palaeolithic levels of the Gran Dolina site in northern Iberia, body size and age of brown trout resulted to be unaffected by temperatures shifts (Blanco-Lapaz et al., 2021). Still, the species was identified only morphologically and ecotypes were not distinguished. Despite this aspect, and the fact that multiple factors (e.g., competition for resources, the selection due to the accumulator agent) might blur the results, fish fossil records have proved to be a unique palaeoecological dataset to study ecological processes on a long-term record but they are still poorly exploited (Blanco-Lapaz et al., 2021).

Species identification is a prerequisite for any study of fish ecology. Taxonomy in archaeological sites has traditionally relied on bone morphology. In contrast, biomolecular techniques such as Zooarchaeology by Mass Spectrometry (ZooMS) provide reliable species identification, through the analysis of species-specific collagen peptides. This technique is minimally invasive, cost-effective and rely on bone collagen type I that can remain preserved for thousands of years (Allentoft et al., 2012; Buckley & Collins, 2011; Holmes et al., 2005). It is particularly effective in fish thanks to the heterotrimetry of collagen type I, meaning that, instead of the two genes that code for the collagen chains in most vertebrates, fish feature three genes (COL1A1 and COL1A2, COL1A3), increasing sequence diversity (Korzow Richter et al., 2011, 2020).

Trout body length can be inferred from vertebral size due to the correlation existing between bone dimensions and body size (Andrews et al., 2022; Prenda et al., 2002; Thieren et al., 2012). Bone formation occurs at different rates according to ambient water temperature, generating thin, denser (darker), winter bands and wider, less dense (lighter) summer bands (Casteel, 1976), that have been used to estimate age in salmonid archaeological samples (e.g., Turrero et al. 2012; Blanco-Lapaz and Vergès 2016). Age estimation, combined with fork length (FL hereafter), in turn, informs about growth rates under different spatial and temporal conditions given that growth has been long known to be dependent on climatic conditions, resources, and selective pressures (Miszaniec, 2021).

The analysis of stable isotopes in bone collagen has been used to infer migratory patterns of ancient populations of salmonids (Guiry et al., 2020; Quinlan, 2023) and to study fish diet (Fuller et al., 2020; Guiry & Robson, 2024). In aquatic ecosystems, carbon and nitrogen stable isotope ratios differ between marine and freshwater biomes (Fry and Sherr 1984, Owens 1987), offering the opportunity to infer anadromy in fish populations (Charles et al., 2004). Notably, the bone collagen of an anadromous trout that feeds on marine prey is δ^{13}C-enriched compared to a resident trout feeding on freshwater prey (Goodwin et al., 2016; Ruokonen et al., 2019).

In the present study, our aim was to study the body, length, age and migratory ecotype of *S. trutta* during the Pleistocene-Holocene climatic transition to assess the impact of climatic changes on intraspecific diversity, in a period of relatively low human impact. The study presented in this paper targeted the Iberian glacial refugium and its northern margins, which is one of the main glacial refugia during the LGM, thought to have been central in the northward post-glacial recolonization of brown trout (e.g., Cortey et al. 2009).
We expect that growth rates of brown trout were lower during the coolest periods of the Pleistocene-Holocene transition than during the warmer periods. A gradient of growth rate values between the core of the Iberian glacial refugium (warmer area) and the northern margin of the refugium is also expected. In modern context, along the Atlantic coast, the fact that all the brown trout populations bear a high diversity life history suggests that this overall strategy is providing an evolutive advantage. Therefore, we also wanted to test whether this diversity was conserved over time and if migratory and resident ecotypes of brown trout were observed together during the Pleistocene-Holocene transition.
The investigation was undertaken by combining multiple approaches, applied together for the first time to Palaeolithic brown trout remains. These includes ZooMS, bone collagen isotopic composition, bone morphometry, sclerochronology to infer species identification, trout ecotype diet, body length, age and growth.

2. Materials and methods

2.1 Study sites and sample selection

Trout vertebrae were obtained from 11 archaeological sites, all karstic caves located in the Cantabrian region of the Iberian glacial refugium and its northern (French) margins, occupied by hunter-gatherers during the targeted period (Table and Fig. 1). Sites occupations dated between the mid-final Upper Palaeolithic (c. 30 to c. 9 radiocarbon kyr B.P.), spanning the Gravettian, Solutrean, Magdalenian and Azilian cultural periods (Table and Fig. 1), a timespan marked by significant climatic fluctuations (brief descriptions of the archaeological sites are provided in Supplementary material). Specimens were initially identified as *S. trutta* based on a morphological classification system that categorizes vertebrae by their vertebral body shape and the feature and density of the lateral pores (Guillaud et al., 2016; Le Gall, 1984). Vertebral size was excluded as an identification criterion to avoid the potential elimination of large anadromous brown trout. A total of 270 vertebrae identified as *S. trutta* (e.g. Fig. 2a) were selected. The vertebrae likely belonged to different individuals as samples were recovered from different meter squares and, layers within the excavations, and vertebrae varied substantially in their dimensions. Species identification was subsequently confirmed through ZooMS, following collagen type I sequencing. All in all, 154 samples were submitted to collagen extraction for carbon and nitrogen stable isotope analysis (SIA), and a subset of 144 bone subjected to ZooMS analyses. Selection for SIA and ZooMS analysis was based on the amount of available bone material and, in the case of ZooMS, on collagen preservation. FL was estimated on all 270 samples. Age inference was performed on that subset of 218 vertebrae identified as brown trout.

Arch. site		Department	GRAV.	SOL.	LM	MM	UM	AZ	
			Pre-LGM	LGM		Post-LGM	Yonger Dryas (YD)	Post-YD	Samples per arch. site
			~30 - 21 kyr B.P.	~21 - 18 kyr B.P.	~18 – 11 kyr B.P.			~11 - 9 kyr B.P.	
1	Taillis des Coteaux	Vienne	26		31	6			63
2	Troubat	Haute-Pyrénées					14	4	18
3	Duruthy	Landes				2			2
4	Aitzbitarte III	Gipuzkoa	6						6
5	Laminak II	Bizkaia					16	6	22
6	Santa Catalina			2		25	11	5	43
7	El Miron	Cantabria		10	15	11	5	2	43
8	Los Canes	Asturias			6		8	10	24
9	La Riera			10		2			12
10	El Buxu			6					6
11	Las Caldas			6		17	8		31
			32	34	52	63	62	27	270

Table 1 Distribution of samples included in the study.

Geographical and temporal distribution of the 270 selected archaeological specimens of salmonids. GRAV., SOL., LM, MM, UM and AZ Stand for Gravettian, Solutrean, Lower Magdalenian, Middle Magdalenian, Upper Magdalenian and Azialian cultural periods, respectively. LGM stands for Last Glacial Maximum.

2.2 Collagen Extraction

Of the 270 samples, a total of 154 archaeological vertebrae of salmonids, recovered from levels dated from 30 to 9 kyr B.P., and 16 contemporary trout (6 anadromous and 10 resident trout) from 8 sites in southern France (reference modern dataset in Supplementary Material) were processed for collagen extraction in the EvoAdapta lab facilities at the University of Cantabria (Santander, Spain). Bone samples (at least 20 mg each) underwent collagen extraction using established protocols (Matsubayashi et al., 2017; Mion et al., 2022). Archaeological bones were cleaned, measured, and photographed prior to destruction. Bones weighing less than 100 mg were demineralized with 0.1M HCl, whereas those over 100 mg were demineralized with 0.5M HCl, with daily acid changes until demineralization was completed. Samples were then rinsed five times with distilled water, soaked in 0.125M NaOH for 30 minutes at room temperature, and rinsed again. Gelatinization was conducted at pH 3 for 48 hours at 75°C. The resulting samples were filtered and decanted into duplicate tubes sealed with parafilm, frozen for 48 hours, and lyophilized for 1-2 days. The 16 samples of contemporary vertebrae were processed using the protocol by Matsubayashi et al. (2017), which includes a degreasing step (6 hours in 1:1 methanol:chloroform solution, followed by two rinses with 99.5% methanol and five washes with distilled water) prior to demineralization. On contemporary sea trout, the *centra* of the vertebral bodies, representing the juvenile stage featuring the freshwater signature, was isolated from the peripheral portion (i.e., the adult life stage), which, in migratory fish, incorporates the seawater signature (Fig. 2b). Collagen extraction was conducted separately on each portion. This approach could not be applied to the archaeological vertebrae due to their friability and poor organic matter preservation.

Of the 154 archaeological specimens processed through the Mion et al. (2022) protocol, collagen of 45 samples was used for ZooMS analysis. For 99 samples (of which 86 that did not yield sufficient collagen following the Mion et al. (2022) protocol, and 13 that were too small to be processed according to that protocol) collagen extraction was repeated to perform ZooMS analysis (Fig. 3). The ammonium bicarbonate (AmBic) method (Section 2.3), adapted for bone samples as small as 10 mg (van Doorn et al., 2011), was employed, as ZooMS requires only a few milligrams of collagen.

2.3 Zooarchaeology by Mass Spectrometry (ZooMS) analysis

A total of 144 archaeological specimens were submitted to ZooMS analysis for species identification. Samples consist of collagen previously extracted (45 samples) or vertebrae fragments (99 samples, average weight ± 12 mg). Samples selection was based on the availability of either collagen extracted for the SIA analysis or osseous material.

ZooMS is a minimally destructive method for taxonomic identification based on collagen peptide mass fingerprinting (Buckley et al., 2009). The extraction and digestion of collagen type I followed published protocols (Buckley et al., 2009; Welker et al., 2015), and was obtained using the AmBic technique (van Doorn et al., 2011), in the lab facilities of EvoAdapta group in the University of Cantabria. All 99 bone samples (~6 – 30 mg) were incubated in 100µL of 50mM ammonium-bicarbonate (NH3CO3, AmBic) buffer at 65°C for one hour. After that, the samples were demineralized in 150 µL of 0.6M hydrochloric acid (HCL) at 4ºC. After neutralization with 50 mM ammonium-bicarbonate (NH3CO3, AmBic), all 144 collagen samples were incubated in 100µl of AmBic at 65 ºC for 1 hour. Then, 50 µl of the resulting supernatant was digested using trypsin (0.5 µg/µL, Promega) between 12 and 18 hours at 37

°C, acidified by adding 1 µL of 10% trifluoroacetic acid and cleaned on C18 ZipTips (Thermo Scientific).

Collagen-digested peptides were then spotted in triplicates on a MALDI Bruker plate, adding αCyano-4-hydroxycinnamic acid as a matrix. MALDI-TOF MS analysis was performed at the University of York (UK) on a Bruker UltrafleXtreme with a mass range of 800–4000 Da.

Spectral triplicates for each sample were merged using R v.4.2.3 (R Core Team, 2023) using the MALDIquant package (Gibb & Strimmer, 2012), following the settings previously described, obtaining .msd files. Taxonomic identification was made manually in MMass v. 5.5.0 (Strohalm, 2023). The table of m/Z values was then compared with a reference library of acknowledged peptide markers (Buckley et al., 2022; Harvey et al., 2018; Korzow Richter et al., 2020).

Among the diagnostic peaks for Salmonids, some are specific to *S. trutta* and *S. salar*. The peptide position COL1α3 568-579 is considered the most reliable marker (Quinlan, 2023), with *S. salar* showing an m/Z peak of 1026 and *S. trutta* displaying an m/Z peak of 996 (Harvey et al. 2018; Buckley et al. 2022; Quinlan 2023, Fig. 4). Another peptide biomarker is identified and helps distinguish the two species: the COL1A2T3 at m/z 3074/3090 and m/z 3084/3100 in *S. trutta* and *S. salar,* respectively.

2.4 Carbon and Nitrogen stable isotope analysis (SIA)

The ecotype and trophic behaviour of past brown trout were investigated using bulk stable isotopes analysis (SIA) of collagen carbon and nitrogen. Collagen was extracted from 154 archaeological specimens and 16 modern specimens (for reference) following the protocol by (Mion et al., 2022). Archaeological samples were selected based on their weight and to ensure a spatiotemporal stratification of the data points. Carbon and nitrogen contents and isotope composition ($\delta^{13}C$ and $\delta^{15}N$), were measured by placing tin capsules containing dry material into an elemental analyser (vario ISOTOPE cube, Elementar, Langenselbold, Germany). The analyser was coupled, via a gas box interface, to a continuous-flow isotope ratio mass spectrometer (Isoprime100, IRMS, Elementar UK, Cheadle, United Kingdom) available at SILVATECH (Silvatech, INRAE, 2018. Structural and functional analysis of tree and wood Facility, doi: 10.15454/1.5572400113627854E12). Elementary gases were analysed by isotope ratio mass spectrometry using Isoprime100 IRMS (Cheadle, United Kingdom). Carbon and Nitrogen contents were expressed as percentages of dry matter, while stable isotope values were expressed as delta values ($\delta^{13}C$ and $\delta^{15}N$, in ‰) relative to V-PDB for C and air N2 for N. Collagen quality was assessed using carbon-to-nitrogen atomic ratio (C:N atomic, 2.9–3.6), which indicates contamination from exogenous carbon or nitrogen (Ambrose, 1990; DeNiro, 1985).

2.5 Ecotype assignment through supervised and unsupervised models

Ecotype classification of archaeological samples was conducted using two approaches: a non-supervised classification based solely on stable isotope data from archaeological specimens, and a supervised classification using stable isotope values from modern brown trout with known ecotypes.

Hierarchical clustering was applied as a non-supervised classification to archaeological samples, using $\delta^{13}C$, $\delta^{15}N$, FL and vertebral dimensions as explanatory variables. Different combinations of these variables were tested in clustering models implemented in R (using the

R *cluster* package), with the number of clusters (k) ranging between 2 to 15 for each combination. The model with the highest Silhouette index was selected for final classification. For the supervised classification, we compiled a dataset of $\delta^{13}C$ and $\delta^{15}N$ values from 140 modern brown trout specimens (63 anadromous and 77 resident), including 59 published and unpublished data points from north-western France (latitude ~+48°N, Charles et al. 2004), 48 published from northern Finland (latitude ~+70°N, Ruokonen et al. 2019) and 16 from southwestern France (latitude ~+43°N, this study). For modern values obtained from fin or muscle tissues, a $\delta^{13}C$ correction of +3.69 was applied (Guiry et al., 2020) to account for tissue-specific isotopic differences. A Suess effect correction was also applied on modern samples with different rates for freshwater and marine environments as the depletion of heavy carbon due to anthropogenic activities differs in these environments (Guiry et al., 2020). For the marine environment correction equation (Guiry et al., 2020), we applied an annual rate of $\delta^{13}C$ decrease for water body (i.e., North Atlantic Ocean) of -0.024 (Quay et al., 2007), and the shape of the exponential curve defined by a decrease in oceanic $\delta^{13}C$ of 0.027 (Gruber et al., 1999; Guiry et al., 2020). The corrected $\delta^{13}C$ values were significantly different between anadromous and resident ecotypes, which was the basis of the ecotype classification. A bootstrapped (1000 iterations) generalised linear model (GLM) with a logistic link function was based on the $\delta^{13}C$ values. Incorporating data from different latitudes allowed the model to mitigate the latitude effect. The model predicted the probability of an individual being anadromous based on its $\delta^{13}C$ value:

Eq. 1

$$P_{(\text{ecotype = "anadromous"})} = 1 \div (1 + e^{-(38.670195 + 2.278882 * \delta^{13}C)})$$

A probability threshold of 0.75 was used for ecotype assignation. The error rate of the model, calculated using the 140 specimens of the modern database with known ecotypes, is 0.021 (false resident rate: 0; false anadromous rate = 0.021). The undetermined-fish rate ($P < 0.75$ for both resident and anadromous classification), calculated on the 140 specimens of the modern database, is 0.071, of which 70% (0.05 of the total) were in fact known to be anadromous and 30% (0.021 of the total) resident. Supervised and unsupervised classifications were combined: samples classified identically by both methods were deemed conclusive, whereas discordant classifications were noted as “undetermined”.

2.6 Fork length, age and growth rate estimation

Through allometric and sclerochronological analyses, we inferred FL, age, and growth rate (GR hereafter) of ancient brown trout.

To develop accurate references for FL and age estimation, we first assembled a reference collection of 51 modern brown trout from different ecotypes and life stages, sampled from various regions along the Atlantic coast. Sampling was stratified according to age (0-9 years), FL (8-76 cm), and sex. Age was estimated through scale annuli counts following Baglinière et al. (2020). Three measurements -height, width, and thickness- were taken on each vertebral centrum, following Guillaud (2014).

Archaeological FL estimation was obtained by generating linear regression models between vertebrae size and FL (Supplementary Material). Incomplete preservation due to mechanical and chemical damage prevented the measure of all the parameters in some archaeological specimens. For such reason, specific allometric equations were generated for each dimension instead of combining them into a single regression model. Separate allometric models were thus constructed for: (a) the atlas; (b) vertebrae without fused neural/haemal spinous processes (NS); (c) vertebrae with fused neural/haemal spinous processes (S), and (d) vertebrae of the urophore complex (i.e., the last three elements of the vertebral column). Within the NS and S groups, the precise position of an archaeological vertebra within the column is impossible to determine, as these cannot be placed in order within each group (Morales, 1984). This limitation explains why, in these two cases, all specimens from the same category were pooled together when calibrating the body-vertebrae regressions. In this case, we used a bootstrapping approach (with 1000 iterations) to draw one vertebral size per individual to fit the regression model.

To predict FL on archaeological vertebrae, considering the available measurements for each sample, the most accurate model for each vertebral type was systematically applied. Previously published models were not adequate for us since some were not only based on both *S. trutta* and *S. salar* modern samples but also more restricted in terms of vertebral categories (Feltham & Marquiss, 1989; Prenda et al., 2002; Van Neer et al., 1999), and measurements (Feltham & Marquiss, 1989; Le Gall, 1984; Prenda et al., 2002; Turrero et al., 2014; Van Neer et al., 1999), and focused on a more limited range of body sizes and/or life stages (Feltham & Marquiss, 1989; Van Neer et al., 1999). Still, the regression models from these publications (Feltham & Marquiss, 1989; Le Gall, 1984; Prenda et al., 2002; Turrero et al., 2014; Van Neer et al., 1999) have been also tested on our reference dataset.

For age estimation, sclerochronological analysis was performed. At least one vertebra for each contemporary fish was photographed with a binocular stereomicroscope using the cellSens Imaging software, with a 1X objective and magnification ranging from 0.5 to 10 depending on the size of the specimen. Images of archaeological vertebrae were captured with APN Nikon D300s camera and Nikon AF-S VR105 mm lens. Multiple images of each vertebra were taken at different focal depths and combined using Combine ZP software, providing detailed views of each vertebra. Pictures of vertebrae from the modern reference collection and the archaeological specimens were analysed using the otolith application method (https://github.com/inrae/otolithe), a tool developed to facilitate collaborative age estimation from pictures. Unlike scalimetry, age estimation from vertebrae is not a common approach in ecology studies of *S. trutta*. We then analysed 40 brown trout with known ecotypes, whose ages had already been estimated from scales, proceeding in two successive steps: a training stage in which readers practise, and a validation stage in which readers assess their success rate. To that end, the modern data set was partitioned into two subsets, a training and a validation set. Two readers were involved, one of whom was an expert in reading salmonid scales (hereafter “expert” reader) and the other without previous experience in sclerochronology (hereafter “candid” reader). First, training was undertaken using a sample of 25 modern vertebrae of known age. Age estimation was performed by counting annuli in vertebrae pictures. Special attention was given to the vertebral pattern and dimensions of juveniles (0+ and 1+) to help interpret the first year of life, one of the most challenging phases. The training process provided a protocol for vertebral annuli counts. A second step consisted in validating the method: readers estimated the specimen's age without prior knowledge of scale-derived ages and compared the ages of vertebrae and scales only at the end of the process. For each modern vertebra, precision (i.e., variation among operators' estimates) and accuracy (difference between real/scale-derived and estimated/vertebra-derived ages) were calculated using, respectively, the coefficient of variation (CV, Vitale et al. 2019) and the mean absolute error (MAE, Hodson 2022). The validated protocol was then applied to archaeological vertebrae. In the latter case, only the coefficient of variation was calculated to assess precision. Finally, estimated ages were compared among readers, and an average and a maximum and minimum age were determined for each vertebra. Maximum and minimum GRs were calculated dividing maximum and minimum FL for minimum and maximum age.

2.7 Statistical analyses

All statistical analyses were carried out using R (version 4.2.3, R Core Team 2023). The object of the analysis was two-fold. First, we investigated significant differences in FL, age and GR correlated to ecotypes, nitrogen signatures, periods, and geographical areas. Second, we assessed the effect of spatiotemporal variation on ecotype and nitrogen signatures. Climatic conditions were also considered. The dating of the major climatic events of the studied period, and correspondence with the study samples is based on the integration of ice cores and marine and terrestrial records (Rasmussen et al., 2014) as illustrated in Table 2.

Non-parametric tests were used in this study as the data did not fulfil the conditions for parametric tests; Spearman's test, corresponding to the parametric Pearson's test, was used to test the correlation between two continuous variables; Wilcox's test, corresponding to the parametric t-Student, tested the effect of a categorical binomial variable on a continuous one, Kruskal-Wallis', corresponding to the parametric one-way ANOVA, tested the effect of a categorical variable with more than two conditions on a continuous variable, and Scheirer-Ray-Hare's test, corresponding to the two-ways ANOVA, tested the effect of two categorical variables on a continuous one.

Cultural period	**Dating (kyrs BP)**	**Glaciation phase**	**Climatic event**	**Climatic conditons**	**GS/I dating (kyrS BP)**
Azilian	11-9	interglacial	Holocene interglacial period	warm	<11.7
Upper magdalenian	13-11	deglaciation	GS-1	cold	12.9-11.7
Magdalenian	18-13	deglaciation	GI-1	warm	17.5-12.9
Solutrean	21-18	LGM	GS-2	cold	23.2-17.5
Upper gravettian	26-21	LGM	GS-3	cold	27.5-23.3
Gravettian	>26	pre-LGM	GS-4	cold	28.6-27.8
Gravettian	>26	pre-LGM	GS-5	cold	32.0-28.9
Gravettian	>26	pre-LGM	GS-6	cold	33.4-32.5

Table 2 Climatic events occurred during the studied period.

The cultural periods of the samples and related dating are reported here together with the corresponding glaciation phase and climatic events and their chronology according to Rasmussen et al. (2014). GS and GI stand for "Greenland stadial" and "Greenland interstadial", representing colder and warmer phases of the North Atlantic region, respectively.

3. Results

3.1 Taxonomic identification

Among the 270 archaeological salmonid samples pre-selected through morphological criteria, collagen spectra generated in 144 via ZooMS allowed species identification of 99 samples (69%). Of these, 72 (73%) were identified as *S. trutta,* and 27 (27%) as *S. salar* (Supplementary material). Due to poor preservation, one-third of the analysed specimens produced incomplete type I collagen spectra, precluding species-level identification. In some cases, unresolved samples exhibited mixed peaks consistent with multiple fish species, including *S. salar* and *S. trutta*.

The combination of ZooMS analysis and vertebral measurements revealed significant differences in the sizes of *S. salar* and *S. trutta* vertebrae for all three measurements (M1, M2, M3) in both S and NS groups (Wilcox test p-value = 1.316e-09 to 0.00440). This suggests that vertebral size could serve as a tool to discriminate species in the 169 unidentified samples. A binomial logistic regression model was applied to this end based on vertebral size range thresholds determined from ZooMS spectra (Supplementary Material). Ultimately, 218 of the initial 270 vertebrae were identified as brown trout.

3.2 Stable Isotopes Analysis: Quality Control

Of the 154 samples subjected to collagen extraction, 129 (84%) yielded collagen. Carbon and nitrogen stable isotope ratios were successfully measured in 69 samples (45% of the analysed sample), 37 of which (24%) met the quality criteria. Among these, 26 were identified as *S. trutta* and 11 as *S. salar*.

All the 16 modern samples that were analysed met the quality criteria.

3.3 $\delta^{13}C$ and ecotype assignment

Hierarchical clustering analysis on the 37 archaeological samples revealed that the optimal clustering model used $\delta^{13}C$ alone with a cluster number (k) of 2. The output of this model, combined with the probability calculated by Eq. 1 revealed a large diversity of ecotypes (Fig. 5, 6a and Table 3), as 6 (23%) brown trout samples were classified as resident ($\delta^{13}C$ range: -24.7‰ to -19.8‰), 7 (27%) as anadromous ($\delta^{13}C$ range: -16.2‰ to -12.3‰), and the remaining 13 (50%) exhibited intermediate signatures ($\delta^{13}C$ range: -18.6‰ to -16.5‰, Fig.5). Among the 11 *S. salar* samples, 6 were classified as anadromous, 2 as resident, and 3 remained undetermined.

The majority (6 out of 7) of brown trout with an anadromous signature were located in French sites (Taillis des Coteaux and Troubat). This regional difference was statistically significant: $\delta^{13}C$ values were overall significantly higher in samples from France compared to Spain (Scheirer-Ray-Hare test, p-value = 0.01), regardless of the climatic condition associated with a given period (Scheirer-Ray-Hare test, p = 0.38).

Arch. site		ID	Cultural period	Climatic phase	Vertebra type	FL (cm)	SRE (cm)	Age (candid)	Age (expert)	Growth rate	$\delta^{15}N$	$\delta^{13}C$	Ecotype
1	**Taillis Des**	TC52	MM	GI-1	NS	46	3	3	2	18.38	9.43	-14.38	anadromous

	Coteaux	TC50	MM	GI-1	S	47	4	3	2	18.73	9.78	-16.54	undetermined
		TC33	LM	LGM	NS	41	3	4	6	8.26	9.41	-14.74	anadromous
		TC54	LM	LGM	S	46	4	4	4	11.45	8.66	-14.80	anadromous
		TC56	LM	LGM	S	40	4	2	2	20.04	7.88	-16.75	undetermined
		TC34	LM	LGM	NS	43	3	3	5	10.69	8.23	-17.45	undetermined
		TC37	LM	LGM	NS	45	3	3	4	12.73	10.02	-17.16	undetermined
2	**Troubat**	MB10	Early Holocene	Holocene interstadial	NS	44	3	-	-	-	16.01	-12.30	anadromous
		MB18	UM	GS-1	NS	46	3	3	-	15.37	13.46	-15.57	anadromous
		MB14	UM	GS-1	S	56	4	3	2	22.32	12.49	-16.17	anadromous
		MB11	UM	GS-1	NS	48	3	-	-	-	8.51	-19.83	resident
		MB12	UM	GS-1	NS	43	3	-	-	-	8.21	-19.94	resident
5	**Laminak II**	LA18	AZ	Holocene interstadial	NS	48	3	3	5	12.07	6.76	-21.02	resident
		LA4	UM	GS-1	NS	56	4	5	4	12.50	13.91	-15.60	anadromous
		LA16	UM	GS-1	NS	47	3	3	2	18.63	13.60	-16.65	undetermined
		LA1	UM	GS-1	NS	50	3	4	3	14.40	12.70	-17.16	undetermined
		LA6	UM	GS-1	NS	49	3	2	3	19.47	12.07	-18.03	undetermined
7	**El Miron**	MR41	AZ	Holocene interstadial	NS	38	3	2	2	18.82	9.18	-17.72	undetermined
		MR1	Solutrean	LGM	NS	50	3	7	5	8.40	10.55	-17.03	undetermined
9	**La Riera**	RR1	Magdalenian	GI-1	NS	49	3	5	3	12.29	7.98	-18.29	undetermined
		RR4	Solutrean	LGM	NS	43	3	6	3	9.61	9.65	-17.43	undetermined
		RR9	Solutrean	LGM	NS	45	3	3	3	15.11	6.08	-18.58	undetermined
		RR6	Solutrean	LGM	NS	41	3	3	3	13.56	5.54	-24.70	resident
11	**Las Caldas**	CL2	UM	GS-1	NS	41	3	5	6	7.47	9.14	-22.47	resident
		CL10	MM	GI-1	S	54	4	4	4	13.41	12.46	-16.87	undetermined
		CL21	Solutrean	LGM	S	36	4	3	3	11.92	7.46	-21.09	resident

Table 3 Stable isotope analysis (SIA) results of brown trout archaeological samples.

SIA results and assigned ecotype of brown trout archaeological samples from this study, for which migratory patterns were studied based on δ^{13}C values. Information on the archaeological site, cultural period, climatic phase, as well as estimated fork length ± RSE (residual standard error), estimated age (candid vs expert), and growth rates are also included.

3.4 δ^{15}N in different environments

δ^{15}N values were significantly different between marine and freshwater environments (Kruskal-Wallis test, p-value = 0.00840. Dunn test: resident-anadromous p-value= 0.0033; anadromous-undetermined, p-value= 0.284; resident-undetermined, p = 0.0413) and ranged between 5.54‰ to 9.14‰ in resident fish, 6.08‰ to 13.6‰ in undetermined fish and 8.66‰ to 16.0‰ in anadromous fish (Table 3 and Fig. 5a). A positive significant correlation between δ^{15}N and δ^{13}C values was also observed (Spearman test, p-value = 0.000270).

Using a Gaussian Mixture Model (GMM) implemented with the *Mclust* R package, $\delta^{15}N$ distributions were analysed for each ecotype. For anadromous trout, the model identified two components (BIC = -35.0) with mean $\delta^{15}N$ values of 9.2‰ ± 0.13, 14‰ ± 1.67. In contrast, resident and undetermined trout exhibited unimodal distributions with mean $\delta^{15}N$ values of 7.6‰ ± 1.43 (BIC = -22.7) and 10.0‰ ± 4.52 (BIC = -61.6), respectively.

3.5 $\delta^{13}C$ and $\delta^{15}N$ on modern samples

The $\delta^{13}C$ and $\delta^{15}N$ values in collagen of the 16 modern anadromous and resident trout analysed for this study ranged between -20.2‰ and -11.1‰ ($\delta^{13}C$ values corrected for the Suess effect) and from 9.2‰ to 13.3‰ ($\delta^{15}N$ values). The average $\delta^{13}C$ values for the external portion of anadromous trout's vertebrae (n = 6), central portion of anadromous trout vertebrae (n = 6), and resident trout vertebrae (n = 10) were -11.7‰ ± 0.35, -14.4‰ ± 0.86, and -17.1‰ ± 2.15, respectively. $\delta^{13}C$ values significantly differed between external portion of anadromous trout vertebrae and resident trout vertebra only (Dunn test p-value = 0.00028). The Dunn test p-value for the comparisons "anadromous trout adult stage – anadromous trout juvenile stage" (Fig. 2B) and "anadromous trout juvenile stage – resident trout" was 0.14 and 0.28, respectively. Average $\delta^{15}N$ values were 12.9‰ ± 0.31 for the external portion of anadromous trout vertebrae, 11.8‰ ± 0.60 for the central portion of anadromous trout vertebra, and 10.8‰ ± 1.02 for river trout. As for $\delta^{13}C$, the difference in $\delta^{15}N$ values were significant only between "anadromous trout adult stage" and resident trout group (Dunn test p-value = 0.00076).

3.6 Fork length, age and growth rate

FL was estimated for 218 brown trout (26 with and 192 without ecotype assignment) and ranged between 23.0 ± 3.4 cm and 60.7 ± 3.4 cm. The models for FL estimation yielded R^2 values ranging between 0.83 (caudal vertebrae, M3) and 0.94 (pre-caudal vertebrae, M1) and residual standard error (RSE) set between 3.4 and 5.6 cm. Previously published regression models resulted to be either less (r-squared between 0.64 and 0.89) or not at all (negative R-squared) suitable for our data (Supplementary material).

Differences in FL across ecotypes were not significant (n = 26, Kruskal-Wallis test, p-values = 0.09 – 0.25, Spearman test [FL- $\delta^{13}C$], p-value = 0.05 – 0.14, Fig. 6b). Average FL in resident, undetermined and anadromous individuals were of 42.7 cm ± 4.81, 46.1 cm ± 4.47, and 47.9 cm ± 5.80, respectively. FL was significantly correlated with $\delta^{15}N$ (Spearman test, p-value = 0.006).

The average FL during colder and warmer conditions stood respectively at 42 ± 4 cm (n = 5) and 48 ± 3 cm (n = 1) in resident trout, 46 ± 4 cm (n = 9) and 47 ± 7 cm (n = 4) in "intermediate" samples, and 49 ± 7 cm (n = 9) and 45 ± 1 cm (n = 4) in anadromous samples. The effect of the climatic variable on FL could not be tested independently on each ecotype due to low number of specimens within each. Overall, no significant effect of climate conditions on FL was detected when analysing the entire dataset (218 samples), which included individuals without ecotype classification. To account for the unbalanced distribution of data points by region and climatic conditions, a subsampling approach was applied. For each 1000 iterations, 24 samples per region and condition combination were randomly selected, and the statistical analysis was repeated. The results showed no significant differences in FL by regions or climatic conditions across these iterations.

Age was estimated for 208 samples and average age ranged between 1 ± 0.7 to 7.5 ± 1.8 years (Fig. 7a). The mean absolute error, calculated from modern samples, was 0.5 years, whereas inter-operator age estimations for archaeological samples yielded a coefficient of variation (CV) of 18.4%.

Average GRs ranged between 6.00 to 30.5. GR for resident, undetermined and anadromous samples were 11.6 ± 1.12, 15.6 ± 1.11 and 15.4 ± 2.11, respectively. The difference is not statistically significant. GR did not change significantly across space and time in the whole dataset. Instead, it appeared to be significantly different in France during different periods. In France (Taillis des Coteaux, Troubat, and Duruthy), average GRs were of 13.1 ± 3.7 (n= 23), 10.8 ± 3.1 (n = 27), 13.9 ± 4.2 (n= 6), 16.9 ± 4.8 (n= 5), and 17.6 (n=1), during pre-LGM, LGM, GI-1, GS-1 and interglacial Holocene period, respectively. We observe a significant GR increment after the LGM until the initial phases of the Holocene (Kruskal-wallis p-value = 0.005, Dunn test p-value [LGM-post-LGM] = 0.003).

4. Discussion

In the present study, we combined a range of biomolecular, chemical and biometric approaches to gather information on *S. trutta* life-history traits during the Pleistocene-Holocene transition. To our knowledge, this is the first time these methodologies are combined to address fish ecology during this period in Europe.

The results suggested a high diversity of *S. trutta* migratory strategies between 26-9 kyr B.P. Our $\delta^{13}C$ SIA values supported the hypothesis of a continuum of ecotypes, aligning with previous studies on contemporary Atlantic populations (Etheridge et al., 2008; Ruokonen et al., 2019). $\delta^{13}C$ values per each ecotype, including a range of "intermediate" values between resident and anadromous ecotypes (-18.6‰ to -16.5‰ in our sample and -19.1‰ to -16.7‰ in Ruokonen et al. [2019]), fell into the variability observed in modern populations. As expected, different ecotypes were featured with significantly different $\delta^{15}N$. No significant correlation between FL and GR was observed among fish with different ecotypes. However, in our dataset, fish of larger body size (FL > 50 cm) were non-resident. A positive significant correlation was found between FL and $\delta^{15}N$. All these results are consistent with modern facultative anadromous populations of brown trout in Norther Europe (e.g., Etheridge et al. 2008). Moreover, our data suggest a gradual GR increment in France after the LGM until the beginning of the Holocene. This is not observed for FL which are unaffected by temperature shifts in agreement with previous studies on early-middle Pleistocene brown trout samples (Blanco-Lapaz et al., 2021).

A crucial point of the study, that contrasts with previous studies of past brown trout ecology (Blanco-Lapaz et al., 2021; Kettle et al., 2011; Turrero et al., 2012) is the species authentication through ZooMS, allowing for an independent assessment of brown trout and Atlantic salmon ecology. ZooMS is able to deal with small-sized samples and has provided robust and reliable discrimination between these two species from the problematic *Salmo genus* once applied to minute fish vertebrae. To our knowledge, this is the first time that ZooMS has been used in Palaeolithic fish samples, offering a promising perspective for future studies. The ZooMS species discrimination on our samples showed that the threshold size criteria applied in previous studies was relevant (e.g., Desse and Desse-Berset 1992). Therefore, despite an only-partially-overlapping vertebral size ranges between *S. trutta* and *S. salar*, the size threshold could be applied when ZooMS analysis is unavailable.

The range of carbon isotope values in archaeological collagen, showed that Upper Palaeolithic *S. trutta* featured resident and anadromous populations. Ecotype assignment was obtained using a non-supervised and supervised classification. The approach was all the more robust, as the archaeological collagen carbon isotope values fell within the same range of values as the modern samples (following a Suess effect correction). Carbon isotope-assigned ecotypes exhibited significantly different $\delta^{15}N$ values as those observed in modern data (Etheridge et al., 2008), reinforcing the validity of the ecotype-assignment method based on carbon isotopes.

Some intermediate values of $\delta^{13}C$ suggest that a substantial proportion of our ancient salmonids featured alternative migration patterns. Indeed, such values could be representative of either i) back-and-forth movements between coastal areas and the river, ii) a short (i.e., less than a year) lapse of coastal habitat occupation or iii) occupation of estuaries and/or low-salinity marine environments. Similar cases -though in lower proportions- have been already documented on modern brown trout and have been interpreted as individuals with an intermediate life cycle, moving back and forth between marine/estuarine and riverine environments (Etheridge et al., 2008; Ruokonen et al., 2019). In our dataset, brown trout with "intermediate" $\delta^{13}C$ values and relatively low $\delta^{15}N$ values, could be interpreted as the result of late-migrations or intermittent periods of migration rather than brackish waters or "fully" anadromous individuals, living under low-salinity conditions at sea, as one would expect higher values of $\delta^{15}N$ for either brackish waters or "fully" anadromous individuals. Other samples, such as MR1, have $\delta^{15}N$, FL, and age values consistent with these two interpretations (i.e., late/intermittent migrants or ecotone denizens). Carbon isotope collagen values rely on collagen turnover, which, to the best of our knowledge, has not been fully documented in fish (Ankjærø et al., 2012; Guiry & Hunt, 2020; Matsubayashi et al., 2017). Therefore, it is likely that movements within periods below a year, which have been reported in the literature for salmonids (Orell et al., 2018), might not translate into full marine signatures. A wide range of intermediate carbon isotopes collagen values might also reflect differences in the duration of the juvenile stage in freshwater prior to migration into seawater, as seen on some modern contexts (Ferguson et al., 2019). Seaward migration appeared to be a size-dependent trait (Ferguson et al., 2019), meaning that growth rate determines the time required to reach a particular size threshold. The age at migration has been found to depend on latitude and river size (e.g., Økland et al. 1993). In Norway, brown trout from southern rivers (latitude 60°/61° N) spend 2-3 years in freshwater whereas this stage rises to 3-6 years in northern rivers (latitude c. 67°N; Økland et al., 1993). The wide range of age estimations in our intermediate and anadromous fish might thus be evidence of varying lengths of the juvenile stage in freshwater before migration (Fig. 6c). Also, in migratory fish, the collagen deposited at the beginning of life will have a different value than that deposited on seawater (Matsubayashi et al., 2017, 2019). Despite our limited sample, our data on vertebrae from contemporary sea trout confirmed that collagen turnover was sufficiently slow to preserve isotopic signatures from different life stages. In our archaeological specimens, alas, separating the juvenile stage from the sea occupation phase was impossible due to the scant collagen yield. While the lifelong collagen was analysed in bulk, a discrimination of freshwater and marine waters occupancy was obtained in line with modern values. Another factor to consider when interpreting SIA $\delta^{13}C$ values for inferring migratory life history is the mixing of freshwater and seawater (e.g., brackish water; Fry 2002; Strøm et al. 2021). Indeed, the assignment of the resident ecotype to two *S. salar* individuals during the post-LGM stage (Fig. 5b) could be attributed to the occupation of a marine habitat under the influence of large freshwater influxes. Substantial freshwater influx into the marine environment due to ice melting between 18–11 kyr cal BP, has been well-documented in palaeoclimatological studies and resulted in lower salinity levels (minimal salinity at -16 kyr B.P.), at least on a local scale (Ménot et al., 2006). Alternative hypotheses of salmon residency appear less likely; in modern contexts, resident salmon exhibit much smaller sizes (e.g., Birt et al. 1991) than the two freshwater-assigned individuals of our study (85 ± 4 cm and 69 ± 4 cm). Therefore, *S. trutta*'s ecotype assignment during the ice-melting period could reflect biases towards freshwater occupancy and/or intermediate life histories.

Such facts notwithstanding, the resident ecotype found during the LGM, provided strong evidence of the presence of this ecotype together with other migratory ecotypes during the transition from the LGM towards milder conditions.

Within the anadromous fish sample, the distribution of $\delta^{15}N$ values revealed two distinct peaks. The ~5‰ difference between them is higher than the typical difference between two trophic levels (Alexandre, 2020; Drucker et al., 2018). This suggests either a high degree of intraspecific trophic diversity in the marine environment or else an occupation of two different habitats at sea with very contrasted trophic food webs. As the specimens from these two groups came from different sites (i.e., 1 individual from Laminak II [$\delta^{15}N$ = 13.9‰], 3 from Troubat [average $\delta^{15}N$ = 14‰ ± 1.82] and 3 from Taillis des Coteaux [average $\delta^{15}N$ = 9.16‰ ± 0.044]), the latter hypothesis seems more probable. Published data evidenced significant $\delta^{15}N$ variation associated with marine locations (e.g., inshore, offshore) and latitude (Chouvelon et al., 2015; Hansen et al., 2012). In addition, our modern reference datasets, including published and newly generated data in our study, revealed differences of 3-5‰ across different locations in Europe. As already claimed in previous studies (e.g., Naito et al. 2013), these considerations underline that caution is needed when interpreting $\delta^{15}N$ values as direct indicators of trophic behaviour without the appropriate context (e.g., $\delta^{15}N$ values of primary producers through amino acids, etc.). For anadromous brown trout, conditions in the marine domain would have been rather variable through time during the period under consideration. The presence of two resident-assigned *S. salar* in two Spanish sites in simultaneous period of times (Las Caldas and Santa Catalina) in post-LGM periods could evidence temporally and spatially restricted large freshwater discharges into the coastal areas.

Regarding fish length and age estimations, our models and protocols were calibrated on more samples (n=51) than in any previous study (e.g., Prenda et al., 2002). In addition, our maximum RSE obtained was 5.6 cm compared to ca. 12 cm of earlier studies (Prenda et al., 2002). This may explain why the published regression models for body length estimation in *S. trutta* tested on our modern brown trout dataset were less accurate than ours. The accuracy and precision of our models were greater in the case of heights (M1) and widths (M2) of vertebral *centra* when compared to thickness (M3). This is partly explained by the fact that, in brown trout, vertebral number is variable and vertebral thickness depends on the number of vertebrae. To the best of our knowledge, this is the first study where the variability within a group of vertebrae of the same type (e.g., NS and S) is accounted for. Our method for annuli count is based on the study of modern brown trout vertebrae and scales. The involvement of an expert and candid reader enabled us to assess the method's viability.

In contrast with some previous studies (Blanco-Lapaz & Vergès, 2016; Desse & Desse-Berset, 1992; Turrero et al., 2012), in our study, while readers could interpret the edge and the core for season of capture and migratory status in modern samples, this could not be achieved for archaeology samples. Further studies might throw further light on this issue.

FL, age and growth rates of our archaeological specimens fell within the range of modern brown trout populations in Europe (e.g., Nevoux et al. 2019, Fig. 7b; Ruokonen et al. 2019). Despite the low number of specimens with an inferred ecotype, the FL range of Palaeolithic brown trout (41-56 cm, 38-54 cm and 36-48 cm for anadromous, intermediate and resident ecotypes respectively) fall within the range of modern northern European equivalents (37-78 cm, 26-59 cm and 27-73 cm for anadromous, intermediate and resident brown trout, in Ruokonen et al. 2019). However, the absence of brown trout on the margins of this range (< 23 cm and > 61cm) might be due to different reasons. For one thing, small vertebrae are more difficult to survive and be recovered. On the other hand, big brown trout might have been less common in the past, although one tend to think that the opposite would hold as a high fishing pressure is known to reduce the maximum body lengths of fish in general (e.g., Limburg et al. 2008). In addition, exceptionally large vertebrae of brown trout might have been assigned to Atlantic salmon, as species identification in the case of non-ZooMS-analysed specimens was based on vertebral dimensions of samples identified through ZooMS. The analysis of further vertebrae of salmonids through ZooMS should help refine our model.

The statistical analyses concerning FL and climatic conditions confirmed the results from previous studies reporting that the climatic shifts during the Pleistocene did not determine variation in brown trout body lengths (Blanco-Lapaz et al., 2021). However, other factors (such as population density, predators, sample accumulator factors, etc) might skew this result.

GRs were significantly affected by climatic conditions in the French region. This effect was not observed in the Spanish brown trout samples, possibly reflecting a lesser contrast between the LGM and deglaciation period temperatures in the Iberian Peninsula, as higher temperatures have been found to be positively correlated with GR in contemporary brown trout populations (Vøllestad et al., 2002). The fact that, unlike GR, FL was not found to be affected by climatic shifts, suggests that during colder periods (or in general harsher conditions), brown trout would have generally reached the smolt stage at a similar body length but a different (i.e., later) age, a hypothesis reinforced by the findings in modern brown trout (Jonsson & L'Abée-Lund, 1993).

In our study, anadromous brown trout were predominantly found in the Northern margin of the Iberian glacial refugia, as originally hypothesised by Roselló Izquierdo (1989) and later by Leal García (2014). This is significant, as the propensity to migrate of anadromous trout enables dispersion and might have driven the (re-)colonisation of post-LGM environments. The presence of anadromous fish, together with improved growth conditions favouring larger sizes, provided conditions for the expansion of trout ranges towards northern Europe during post-glacial times (Cortés-Guzmán et al., 2024; Ferguson, 2006; Jarry et al., 2018). The hypothesis that post-LGM colonisers derived mostly from the northern margins of the glacial refugium could be tested through genetic analysis.

The present study provides insights into fishing activities during the Pleistocene-Holocene transition. Although no analysis of butchery marks was undertaken, most of the samples from which our specimens were selected were probably accumulated by humans, as proved by the material culture and archaeozoological analyses of the macrofauna. Still, the role played by non-human fish accumulators in northern Iberian archaeological sites has not been systematically pursued through taphonomic analyses with the exception of Laminak II (Roselló and Brinkhuizen 1994). The presence of anadromous fish on inland sites, in turn, suggests that these were likely targeted by nomadic humans acquainted with the seasonal migrations of the animals. If present-day trends constitute valid analogues for interpretation, then one may assume that anadromous migrations coincided with the colder months of the year. This would turn salmonids into a crucial fall-back resource when alternative foodstuffs were scarce, and more so if their meat could be processed for delayed consumption. Year-round availability of resident freshwater fish, in turn, would make these reliable targets and a dietary supplement when anadromous fish were absent or scarce. Our study reveals that this dual reliance on both anadromous and resident fish hints at a strategic resource utilisation approach to optimising fish availability throughout the year.

Even though sampling of the archaeological collection was stratified to include different vertebral sizes, biases due to the way archaeological excavations have been traditionally carried out and a size selection due to the accumulator are intrinsic to the sample. Moreover, part of the present study relied on collagen extracted from fish vertebrae. The requirement of at least 40 mg of tissue for reliable stable isotopic results restricted the incorporation of the smallest fish, potentially introducing additional size-related biases into the datasets. Although 57% of the samples collected were above that weight threshold processed for collagen extraction, only ~10% yielded collagen that meet the quality criteria required for ZooMS and SIA. Indeed, low success rates have been observed in similar archaeological fish studies (e.g., Robson et al. 2016). The small and porous nature of Salmo sp. vertebrae, as well as the mineral and collagen structure of fish bones in general, may significantly influence collagen preservation (Szpak, 2011). Samples used in the present study were far older than those of equivalent studies in fish, and this might seriously compromise collagen preservation.
One final major issue with the present study concern the limited number of samples with reliable SIA restricting the power of the statistical analyses. This limitation is more relevant when one considers the multiple factors to test for (e.g., climatic events, archaeological contexts, cultural periods, etc.). Our samples cover a long temporal window, during which multiple climatic events took place, and derive from a good number of archaeological sites. Whereas this aspect represented an interesting novelty of our study and was originally planned, it also posed challenges in terms of interpretation due to the restricted number of specimens with meaningful SIA results. Grouping climatic events into broad categories (i.e., cold vs warm; Table 2) and archaeological sites in terms of macro-regions (France vs Spain), helped us with interpretation the results but risks over-simplifying matters.

5. Conclusion

The present study provides insights into brown trout phenotypic intraspecific variation during the Pleistocene-Holocene transition, an important period for life's evolutionary history. Our findings underscore the adaptative response of brown trout to environmental changes, thanks to its high phenotypic diversity, in terms of life-history strategies, diet, and size at maturity that we found held on throughout the period under consideration. This resilience of brown trout during the LGM might have been advantageous for human populations, by providing a reliable source of proteins year-round, but in particular during lean times.

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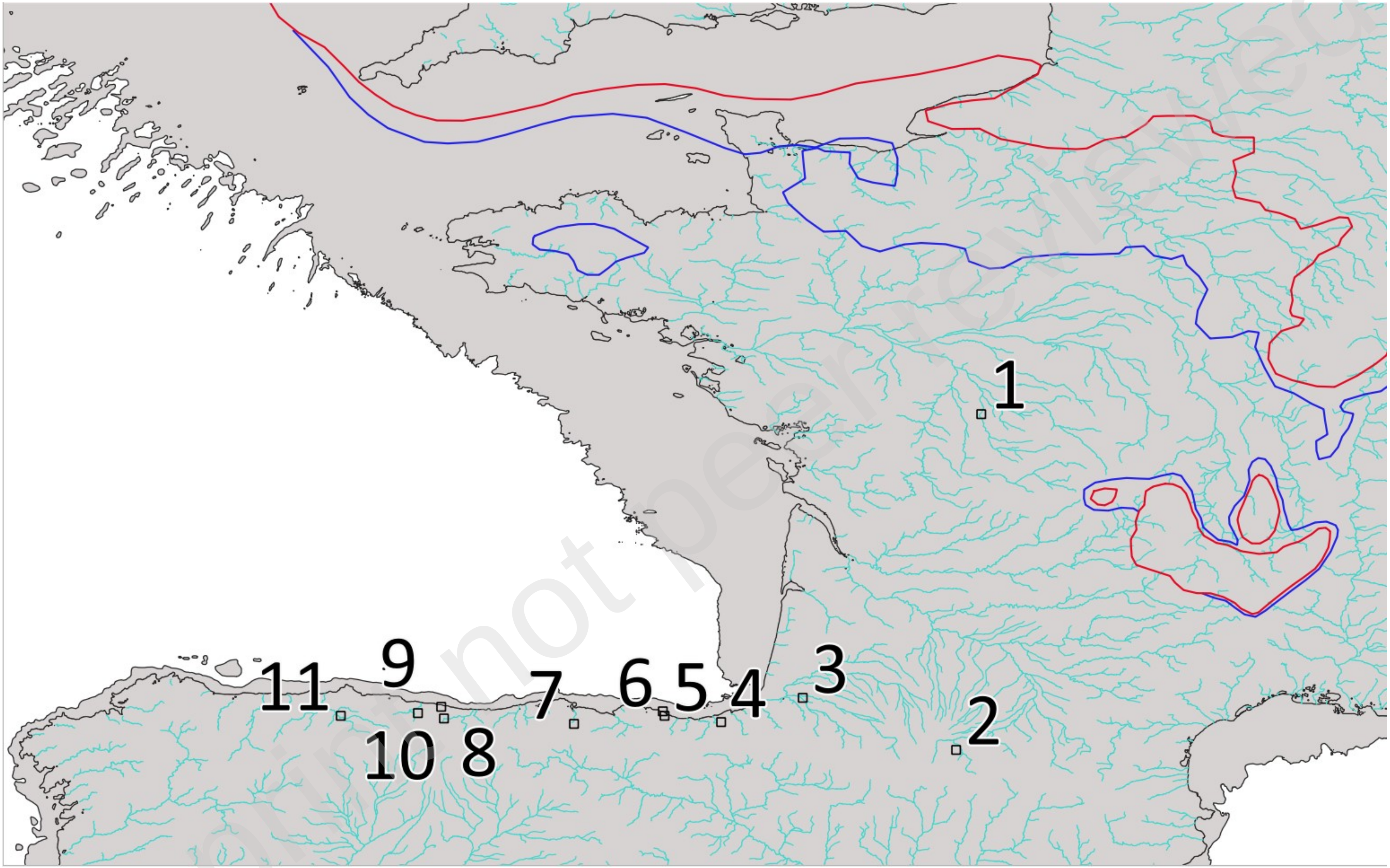
1
2
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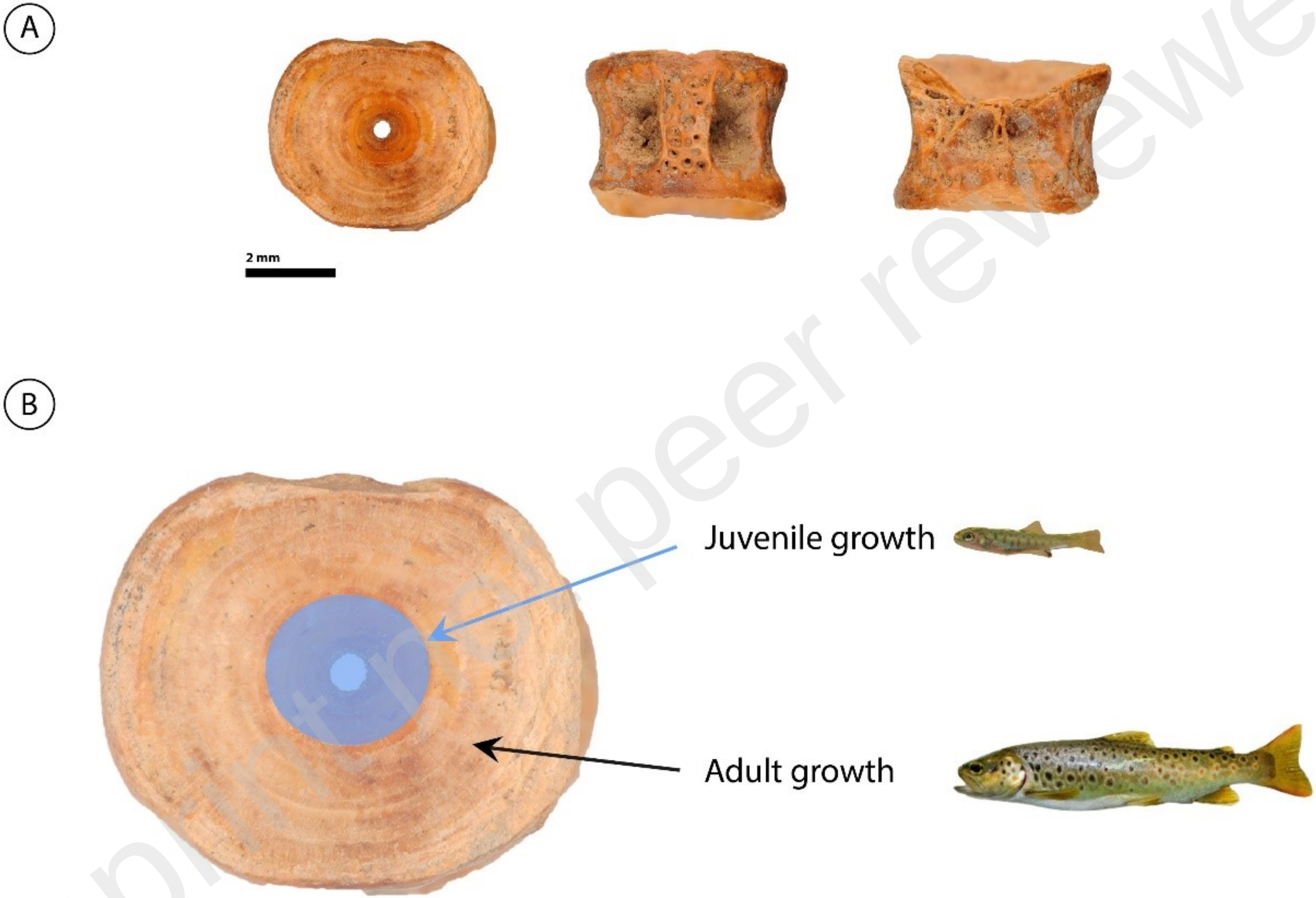
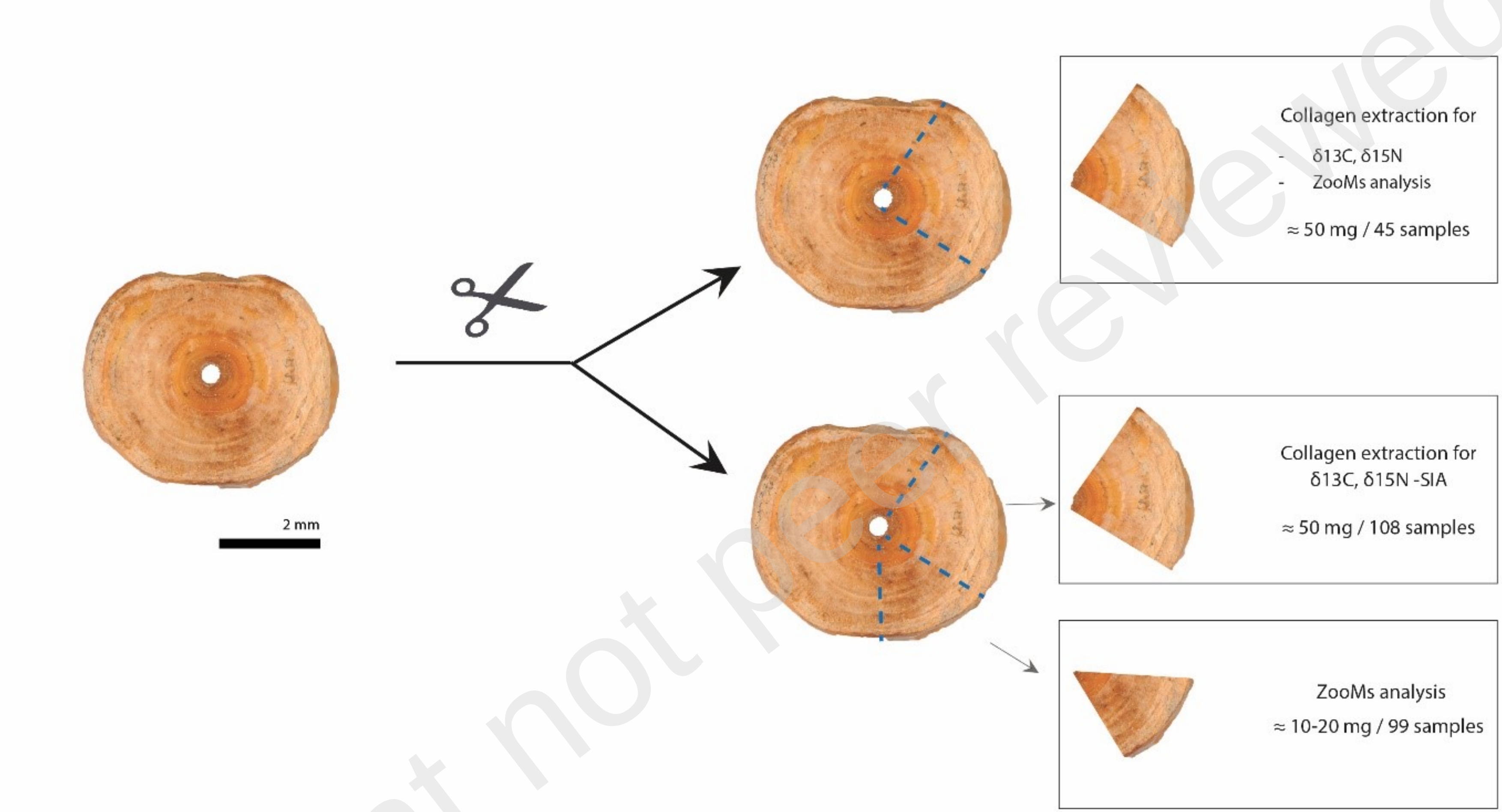
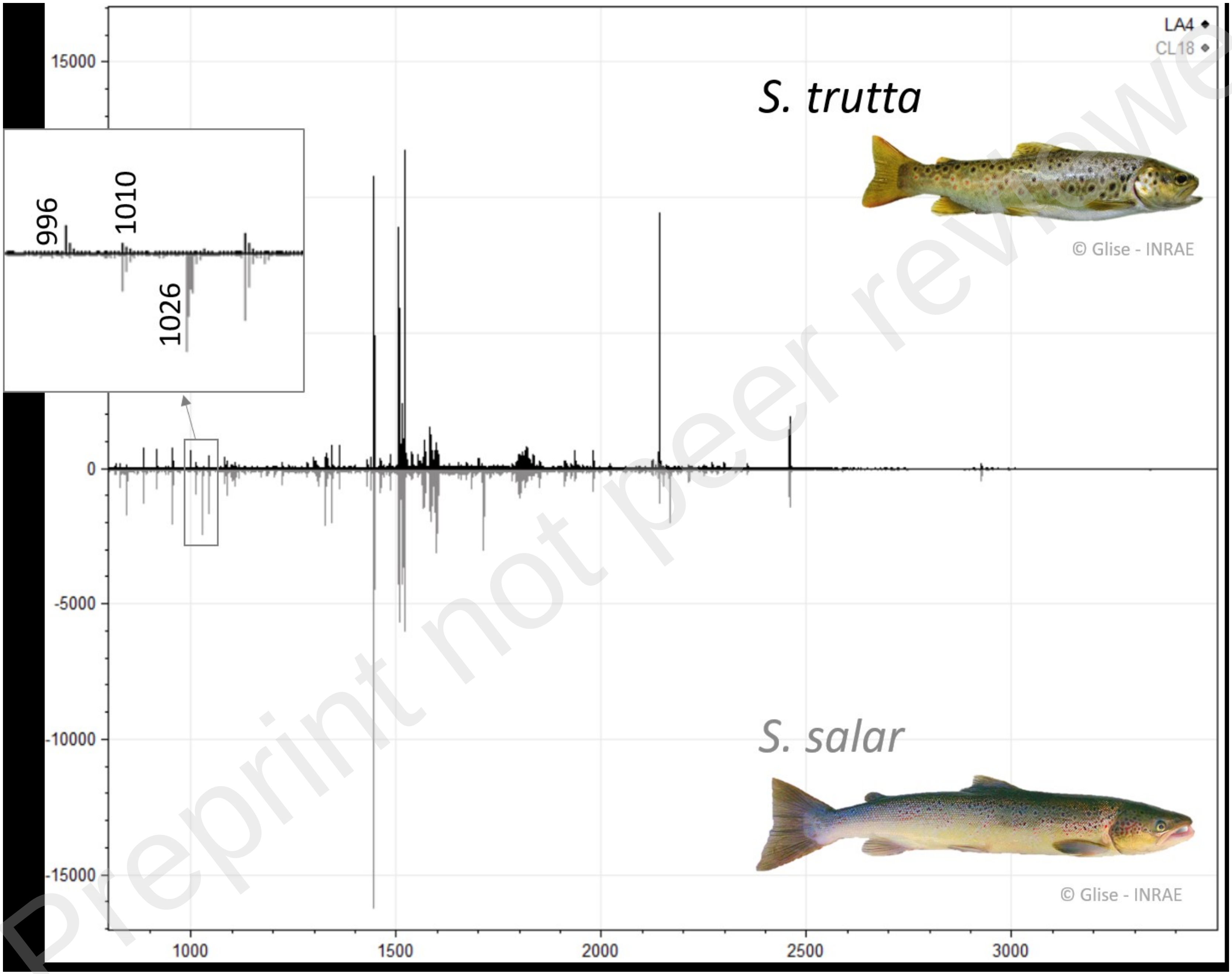

A
2 mm
B
Juvenile growth
Adult growth


2 mm
Collagen extraction for
- δ13C, δ15N
- ZooMs analysis
≈ 50 mg / 45 samples
Collagen extraction for
δ13C, δ15N -SIA
≈ 50 mg / 108 samples
ZooMs analysis
≈ 10-20 mg / 99 samples



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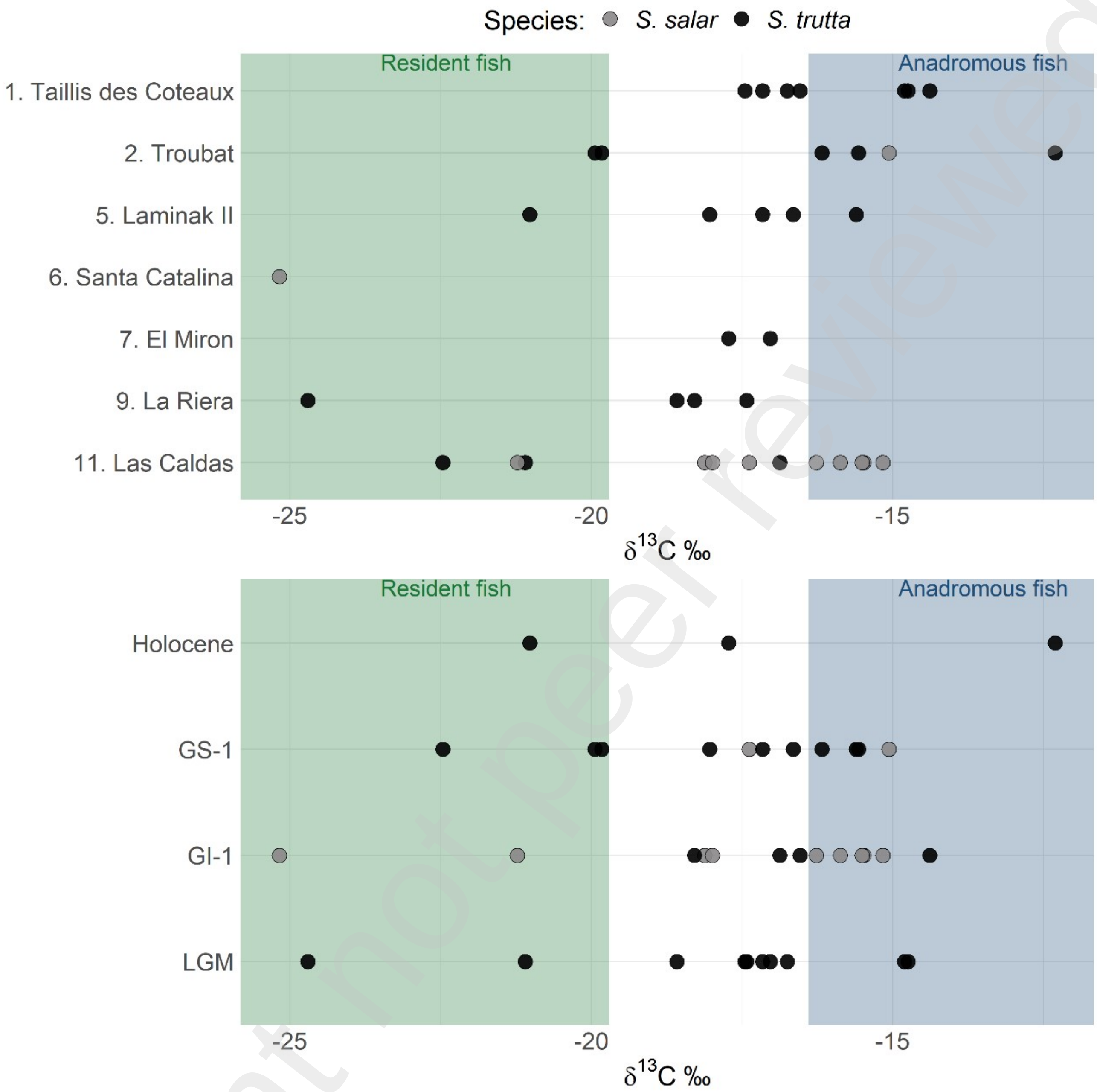
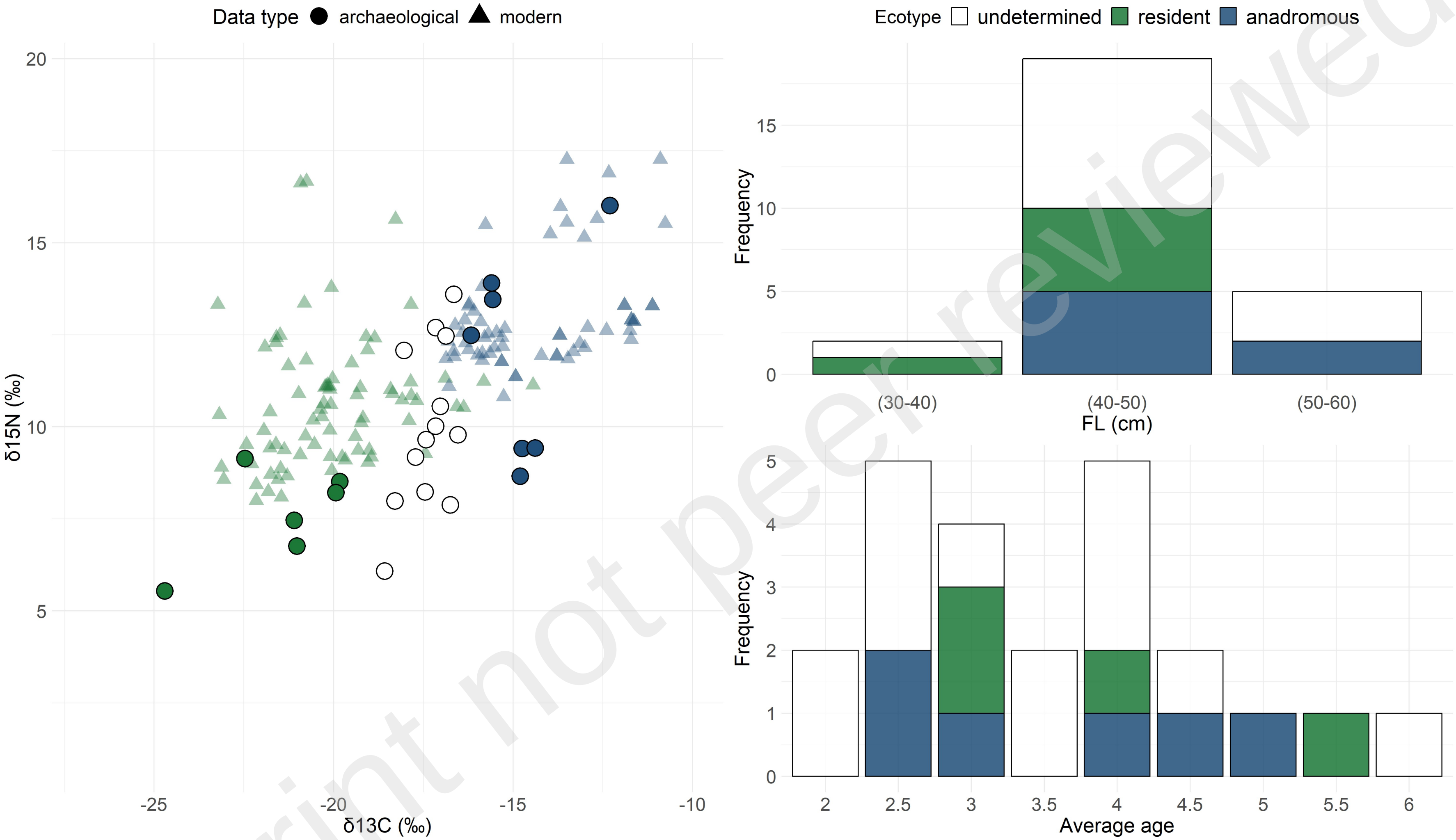

Species: ● S. salar ● S. trutta
Resident fish
Anadromous fish
1. Taillis des Coteaux
2. Troubat
5. Laminak II
6. Santa Catalina
7. El Miron
9. La Riera
11. Las Caldas
-25
-20
-15
δ^{13}C ‰
Resident fish
Anadromous fish
Holocene
GS-1
GI-1
LGM
-25
-20
-15
δ^{13}C ‰


Data type ● archaeological ▲ modern
δ15N (‰)
20
15
10
5
-25
-20
-15
-10
δ13C (‰)
Ecotype ☐ undetermined 🟩 resident 🟦 anadromous
Frequency
15
10
5
0
(30-40)
(40-50)
(50-60)
FL (cm)
Frequency
5
4
3
2
1
0
2
2.5
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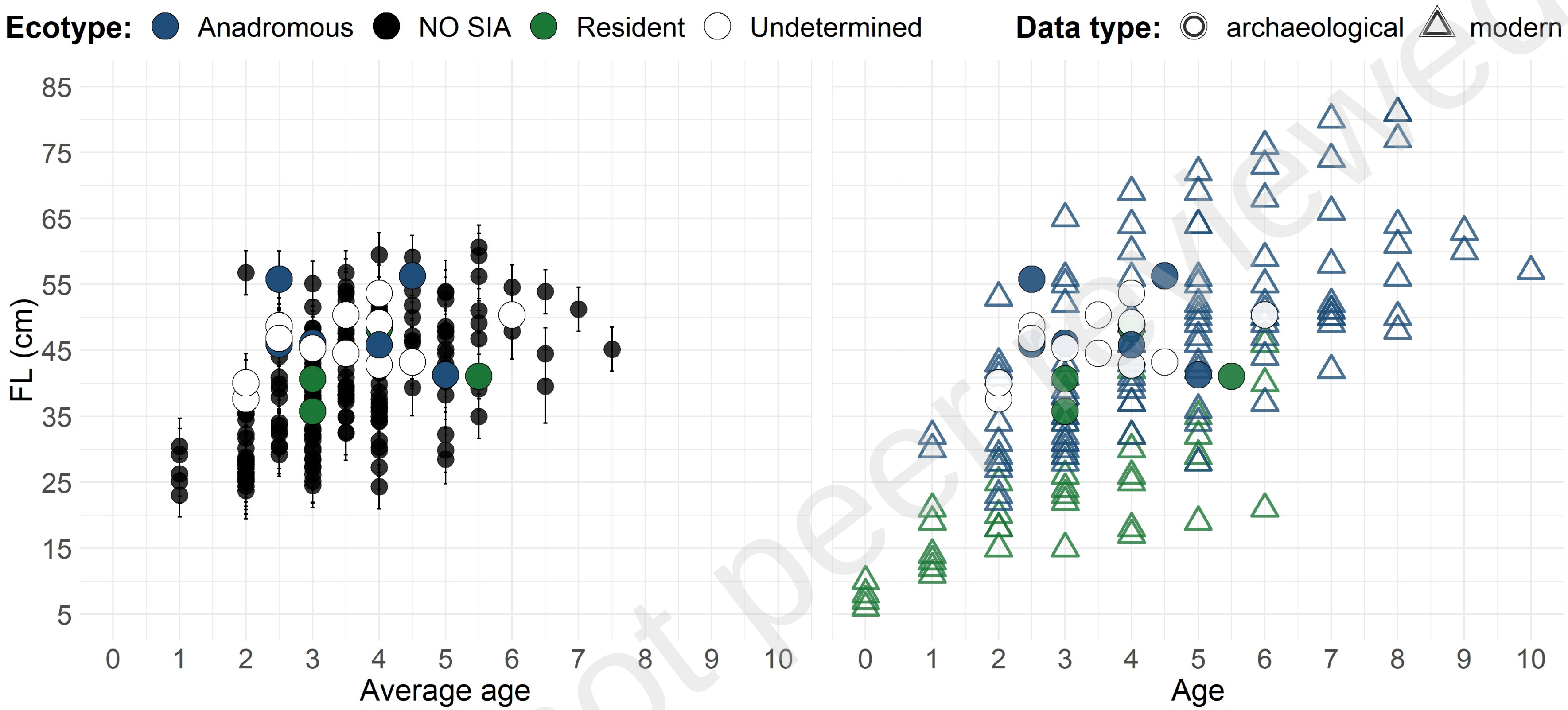
4
4.5
5
5.5
6
Average age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● NO SIA
● Resident
○ Undetermined
Data type:
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△ modern
FL (cm)
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75
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55
45
35
25
15
5
0 1 2 3 4 5 6 7 8 9 10
Average age
0 1 2 3 4 5 6 7 8 9 10
Age

Fig. 1 Geographical distribution of archaeological sites included in the study.

Geographical distribution of the eleven archaeological sites included in the study: 1. Taillis Des Coteaux, 2. Troubat, 3. Duruthy, 4. Aitzbitarte III, 5. Laminak II, 6. Santa Catalina, 7. El Mirón, 8. Los Canes, 9. La Riera, 10. El Buxu, 11. Las Caldas. The grey shading represents the coastline during the Last Glacial Maximum (LGM), while the blue and red lines indicate the extent of the discontinuous/sporadic permafrost and continuous permafrost during the LGM, respectively (Lambeck et al., 2014; Opel et al., 2024; Stadelmaier et al., 2021).[1]

Fig. 2 Vertebrae of brown trout.

A. Archaeological vertebrae of a brown trout from the archaeological site of El Mirón: transversal, dorsal and ventral view of the vertebral body. B. Sampling strategy adopted for stable isotope analysis (SIA) on six contemporary anadromous brown trout vertebrae: juvenile and adult portions were analysed separately.

Fig. 3 Sampling of archaeological vertebra.

A portion of 50 mg of vertebral bone was used for collagen extraction for $\delta^{13}C$ and $\delta^{15}N$ stable isotope analysis (SIA) for 154 archaeological vertebrae through the protocol by (Mion et al., 2022). A few mg of the obtained collagen was used for ZooMS analysis for 45 samples. On an additional 99 samples, a further fragment of bone of ~10 mg was used for collagen extraction for ZooMS analysis through the protocol by (van Doorn et al., 2011).

Fig. 4* S. salar *and* S. trutta *ZooMS major diagnostic peaks.

The peptides of collagen type I, revealed through ZooMS, of two Salmonids analysed in the present study are shown in the picture as they appear in the Mmass software: the peaks of a brown trout sample from Laminak II in black, and of an Atlantic salmon from Las Caldas in grey.

Fig. 5 Carbon isotope values distribution for* S. trutta *and* S. salar *per site (upper panel) and per period (lower panel).

Periods are assigned according to Table 1. GS and GI stand for Greenland stadial and interstadial and represent colder and warmer phases of the North Atlantic region, respectively.

Fig. 6 Ecotype, fork length and age of 26 Upper Palaeolithic brown trout.

S. trutta *ecotype assignment and relative stable isotope analysis values (left panel), fork length (FL) range (upper right panel) and age range (lower right panel) of fish (N=26). The modern reference dataset (N = 140) used for ecotype classification through the supervised model is represented in the left panel.*

Fig. 7 FL and age distribution of 218 Upper Palaeolithic brown trout and contemporary brown trout.

The left panel displays FL distribution (with relative error) and age distribution of 218 brown trout archaeological samples, including those without ecotype assignment (black). The right panel shows FL and age distribution of contemporary resident (green) and anadromous (blue) brown trout previously published (Nevoux et al., 2019) together with the 23 (6 anadromous, in blue, 4 resident, in green, and 13 intermediate, in white, archaeological brown trout for which ecotype, FL and age was estimated in this study (Table 3).

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[1] Map lines delineate study areas and do not necessarily depict accepted national boundaries.