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Title: **The curious case of the Mesolithic Iberian dogs: an**

archaeogenetic study

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Abstract:

We investigated the genetic composition of six *Canis* remains from western Iberia, directly radiocarbon dated to 7,903-7,570 years (cal BP). They were identified as dogs via their archaeological and depositional context, osteometry, and a high percentage of aquatic diet shared with humans. For comparison, genetic data were obtained from an additional 37 Iberian dog remains from the Neolithic to Late Antiquity, as well as two Palaeolithic and a Chalcolithic *Canis* identified as wolves. Previous data indicated that dog mtDNA haplogroup A (HgA) is prevalent in extant European dogs (>50%), in the Near East and Asia, but rare or absent (<10%) in European *Canis* older than 3,000 years (cal BP). We found a high frequency (83%) of dog HgA in Mesolithic Iberian dog remains. This is the first report of a high frequency of dog HgA in pre-Neolithic Europe. We show that, contrary to the current view, *Canis* with HgA did not necessarily arrive in Europe from East-Asia. This phylogeographical difference in HgA frequency demonstrates that genetic differentiation was high prior to, or as a consequence of, domestication which may be linked with pre-Neolithic local processes for Iberian wolf domestication. Our results emphasize that knowledge of both ancient wolves' and early dogs' genetic profiles from the European periphery should improve our understanding of the evolution of the European dog.

Keywords: Dog, wolf, domestication, Iberia, zooarchaeogenetics, ancient DNA, mitochondrial DNA

1. Introduction

The geographical centres for wolf domestication is still much debated (Botigué et al., 2017; Frantz et al., 2016; Shannon et al., 2015; Thalmann et al., 2013; Wang et al., 2016). Unlike other domesticated animals like cattle, sheep, goats and pigs, wolves were domesticated before the Neolithic by hunter-gatherers (Clutton-Brock, 1999; Davis and Valla, 1978; Morey and Jeger, 2015). In Europe, little is known about the genetic diversity of wolves prior to their domestication (Fan et al., 2016; Pilot et al., 2014, 2010), and concerning early dogs the little we know is limited to central, northern and eastern regions (Botigué et al., 2017; Frantz et al., 2016). Peripheral areas such as the Iberian Peninsula remain essentially *terrae incognitae*. Understanding the complex genetic origins and diversity of extant dogs in Europe requires a major re-evaluation of the genetic characteristics of ancient populations across the whole continent. This re-evaluation will highlight the role of the genetic structure of ancient wolf populations in the domestication process and in shaping present-day genomic diversity of dogs.

In the Iberian Peninsula, the oldest evidence for the presence of dog is a humerus dated to 18,000-12,000 cal BP (dated by context) in Erralla (Spain) (Vigne, 2005). In Portugal, the oldest dog remains are from the Mesolithic period (c. 8,000-7,500 cal BP), which were found in shell-middens in the Tagus and Sado palaeovalleys, namely in large archaeological sites such as Cabeço da Amoreira and Cabeço da Arruda in Muge (Tagus valley) and Poças de São Bento (Sado valley) (Arias et al., 2016, 2015; Detry and Cardoso, 2010). Another dog was recently identified in Vale Boi, Algarve (this study). During the Mesolithic, dogs were often buried with humans (e.g. Skateholm, Sweden;(Larsson, 1990). Since its discovery in the 19th century, some 200 human skeletons have been exhumed at the Muge shell-middens together with at least one dog

(Detry and Cardoso, 2010). In 2012, archaeologists discovered a dog burial at Poças de São Bento (Sado), where human burials had also been found (Arias et al., 2016, 2015).

In the Natufian period in the Middle East, a human burial clearly associated with a juvenile dog was also found at the Natufian site of Ein Mallaha (Davis and Valla, 1978; Tchernov and Valla, 1997). These cases provide strong evidence for an affectionate rather than a gastronomic relationship between humans and dogs (Davis and Valla, 1978). Before the Mesolithic, human burials were rare and dog burials are so far unknown.

In a genetic study using ancient *Canis* (dogs and wolves) samples covering the last 15,000 years, Franz et al. (Frantz et al., 2016) found that mitochondrial DNA (mtDNA) HgA haplotypes were absent from all European regions sampled preceding 6,700 years cal BP, but were present in East Asia and the Near East. One of the few exceptions bearing a HgA haplotype, was a single sample from southern Italy (Romanelli cave, PIC3, 9,670 ± 40 years BP) which could not be clearly identified as a dog or a wolf (Sardella et al., 2014; Verginelli et al., 2005). It was proposed then that HgA increased in frequency and perhaps arrived in Europe mainly as a consequence of the Neolithic or even at a later time.

Zooarchaeogenetics can aid our understanding of the process of domestication, evolution and livestock improvement in the past (e.g. (Davis et al., 2012; Niemi et al., 2018; Svensson et al., 2007)). As a result of the process of domestication, most large mammals such as the wolf (ancestor of the dog), aurochs (ancestor of cattle) and wild boar (ancestor of the pig) decreased in size (Darwin, 1885). Moreover, dogs generally have shorter snouts giving them paedomorphic characteristics (Tchernov and Valla, 1997), which are especially reliable features to identify their domesticated status. These differences in size and cranium shape are the main criteria used by zooarchaeologists to distinguish dog from

wolf remains. The same trend was indeed found in Portuguese Mesolithic *Canis* with their smaller size and shorter crania (Detry and Cardoso, 2010).

MtDNA has been useful for addressing dog intraspecific evolutionary questions (e.g. (Frantz et al., 2016; Leonard et al., 2002)). In what concerns extant dogs and wolves from the Iberian peninsula, their mtDNA sequences are generally well differentiated (Pires et al., 2017a), but their former genetic structure, back when the first dogs first appeared, is yet unknown.

In this study we aim to understand the genetic composition and differentiation of the earliest dogs in Mesolithic western Iberia compared to other contemporary European dogs. The Mesolithic shell-middens in the Tagus and Sado valleys in western Iberia, whence most of our dog remains were collected, are among the most important archaeological sites in the world of this period. This is partly due to the abundance of human skeletons recovered in many of these sites (>300) (Cunha et al., 2003). The dog is, so far, the only known contemporary domesticated animal. Other domesticated animals associated with the subsequent Neolithic period - sheep, goat, pig and cattle- as well as ceramics, and domesticated plants such as wheat and barley were brought to the western part of the Iberian Peninsula (i.e., c. 7,500 years cal BP; (Davis and Simões, 2016; Martins et al., 2008; Zilhão, 2001).

By contributing zooarchaeogenetical data from western Iberian Mesolithic dogs, including directly radiocarbon-dated remains and isotope analyses, we offer a new perspective on the origin and evolution of the dog in Europe.

2. Materials and Methods

2.1. Zooarchaeology

2.1.1. Archaeological samples

Below we describe in detail from the oldest to the latest, the Palaeolithic, Mesolithic and Chalcolithic *Canis* remains studied and which are the core of this zooarcheogenetical study:

Palaeolithic *Canis* samples

The root of a *Canis* tooth (LYEP46) was found at the Furninha cave (Peniche, Portugal) by Nery Delgado in the 19th century and its context was dated via the Uranium-Thorium method, to 80,886±31.265 years BP (Middle Palaeolithic, interglacial period). It was not possible to collect odontometric data from this sample due to its fragmented state.

Another *Canis* mandible (LYEP44), with pre-molars 3 and 4 *in situ*, was collected at Algar the João Ramos cave (Alcobaça, Portugal) by Romão de Sousa in 1909. It was indirectly dated to the Upper Palaeolithic (ca. 14,000 years BP) based on the dating of another bone from the same context (Antunes et al., 1989; Cardoso, 1993). A recent attempt to radiocarbon date LYEP44 directly was unsuccessful due to the lack of collagen, which also prevented the collection of isotopic data (a signature of its diet). Measurements of its lower fourth pre-molar length is 13.7 mm and fall within the range of variability of the Palaeolithic wolves (glacial and interglacial) ((n=23) [13.2-17.1] mm) (Cardoso, 1993). The Algar João Ramos cave was not occupied by humans during the Upper Palaeolithic and only provided archaeological remains dated to the Neolithic. This find was covered by a red sediment typical of the Pleistocene layers and very different from the Neolithic ones with their dark colour. All of the above led us to identify this specimen as a wolf.

Mesolithic *Canis* samples

A *Canis* specimen (LYEP3) was found during the 2003 excavations in Vale Boi (south of Portugal) directed by Nuno Bicho (Bicho et al., 2013, 2012). The highly-fragmented condition of this specimen prevented any measurements from being taken. It was found in an archaeological site, i.e. in a context with evidences of past human activities, including a human tooth dated from the same time span.

Some other isolated remains of *Canis* were found in Muge (Cabeço da Amoreira) during the 1930's excavations directed by Mendes Correa. These are part of the collections of the Museu de História Natural e da Ciência of the Universidade do Porto, Portugal, and include a right proximal humerus (SEP002) described herein. Its epiphysis was fused to its diaphysis indicating an age at death greater than 12 months (Habermehl, 1975).

A 2012 excavation at Cabeço da Amoreira uncovered another Mesolithic *Canis* - a maxilla fragment with three teeth *in situ* (LYEP75) (Fig. 1A). It was discovered during excavations directed by Nuno Bicho and is stored at the Universidade do Algarve in Faro, Portugal. The presence of both the deciduous upper fourth pre-molar (dP^4) and the permanent upper fourth pre-molar as well as the upper first molar indicates that the animal was probably around five months old when it died.

A *Canis* mandible (LYEP68B) uncovered by Jean Roche also at Cabeço da Amoreira (Muge), in the 1960's excavations and currently housed in the Museu Geológico, in Lisbon, Portugal, was at least four years old when it died (using the Horard-Herbin's wear pattern scale for teeth (Horard-Herbin, 2001)).

In 2000, another almost complete *Canis* skeleton (LYEP68A) from Cabeço da Arruda (Muge) was identified at the Museu Geológico in Lisbon, Portugal, (Fig. 1B) which had been excavated in 1880 by Carlos Ribeiro, the geologist who first discovered the Muge shell-middens (Tagus valley). The absence of bones with unfused epiphyses in this skeleton indicates that it was older than two years at the time of death. Estimation of the shoulders height, using Koudelka constants, indicates a shoulder height of around 48.5 to 51 cm –a medium sized dog (Detry and Cardoso, 2010).

In 2012, Pablo Arias and Mariana Diniz found an almost complete *Canis* skeleton (LYEP74) at Poças de São Bento, a Mesolithic shell-midden located in the Sado valley, now housed in the Museu Nacional de Arqueologia in Lisbon, Portugal. This skeleton was found *in situ* (Fig. 1C), suggesting that it was deliberately buried, perhaps as part of a ritual (see (Arias et al., 2015 and Arias et al., 2016 for a preliminary view of the context of this finding). This animal had erupted permanent teeth indicating an age at death greater than five months. The presence of a visible suture line in the distal tibia indicates that the animal was probably around 15 months old (Habermehl, 1975). The maintenance of the dog buried in its original position prevented a complete osteometric characterization.

Fig. 1 – Photos of some Mesolithic Iberian *Canis* remains: Fragment of a *Canis* right maxilla (LYEP75) from Cabeço da Amoreira, Muge, Portugal (A) and from the two most complete *Canis* skeletons - LYEP68A from Muge – Cabeço da Arruda, Tagus shell middens (B) and LYEP74 – from Poças de S. Bento, Sado shell middens (still included in the sediment) (C). Photos by José Paulo Ruas.

Chalcolithic *Canis* sample

A single *Canis* specimen from the Chalcolithic (LYEP27), a fragmented left lower carnassial tooth, was recovered during the 1999 excavations at Penedo do Lexim (Portugal). It was only possible to measure its maximum width, which is 13.4 mm indicating that it probably belonged to a wolf (Moreno-Garcia et al., 2016). The indirect radiocarbon date for a specimen of *Sus* from the same stratigraphic unit indicates that this wolf was probably approximately 4,085-3,856 years old (cal BP).

Other archaeological *Canis* samples

The other 37 archaeological samples included in this study for comparison purposes were the subject of other osteometrical and genetic studies. References to those studies and respective results can be found in Sup. Table S5.

2.1.2. Radiocarbon dating

All six Mesolithic *Canis* remains were directly radiocarbon dated (cal BP, ± 95% confidence) using OxCal v. 4.2 (Ramsey, 2009) with IntCal13 and Marine13 Curves (Reimer et al., 2013) (see Fig. 2). Different ΔR values were used: 140±40 ^{14}C (Cabeço da

Arruda and Cabeço da Amoreira shell-middens, Muge, Tagus Valley) (Martins et al., 2003), -100±155 ^{14}C (Poças de São Bento shell-midden, Sado Valley) and 95±15 ^{14}C (Cabranosa and Padrão, Algarve region) (Monge Soares et al., 2016). The proportion of aquatic protein in their diet was taken into account in the calibration process (Ambrose, 1993).

2.1.3. Osteometric analysis

Canis bones and teeth were measured with a digital caliper (Mitutoyo Digimatic caliper, CD-8"C model with a precision of 0.01 mm) and followed the criteria of von den Driesch (von den Driesch, 1976) in order to ascertain their wild/domestic status prior to undertaking DNA analysis. The standard measurements taken were breadth *versus* length for the 4th upper pre-molar tooth (PM^4); length of the lower 1st molar tooth (M$_1$); and the proximal width of the humerus. These measurements can reflect the reduction in size and muzzle shortening, which are features frequently associated with domestication.

According to Bergmann's rule (Bergmann, 1848) (see also (Salewski and Watt, 2017), mammals and birds tend to vary in size inversely with the temperature of the environment. Thus, species or groups of closely related taxa tend to be larger in colder regions and smaller in warmer ones. To avoid geographical bias in size we compared measurements of the Mesolithic *Canis* with those from reference specimens in collections of extant dogs and wolves and ancient wolves from Portugal. Data are presented in Sup. Table S1 and Fig. 3.

2.1.4 Statistical analysis

In order to assess how potentially different, the Mesolithic *Canis* remains are from dogs or wolves, we compared Iberian dogs and wolves from other periods. Namely, three

different sets of samples of 85 extant wolves, 7 Upper Palaeolithic wolves, and 16 Middle Palaeolithic wolves. As for extant dogs, a collection of 38 skeletons was used for the same purpose (Sup. Table S1).

Based on the standard measurements described above, Student's t-tests were used whenever parametric assumptions were met while Mann-Whitney and Kruskall-Wallis tests were applied whenever samples did not meet such assumptions. The effect size (Cohen's d for t-tests and r for Mann-Whitney tests) was then calculated for every statistically significant result ($\alpha = .01$) thus providing a measure of the magnitude of such differences.

Finally, we joined all the zooarchaeological evidence together and used Bayesian analyses to assign the Palaeolithic, Mesolithic and Chalcolithic samples to either the dog or wolf category. For each sample and for each data type available (direct dating, osteometry and isotope composition) we computed the likelihoods for the observed data under the hypothesis of being a dog (HD) and under the hypothesis of being wolf (HW). A joint likelihood for all data types was obtained by multiplication of the likelihoods under each hypothesis, for each *Canis* remain. The likelihood ratio (LR) for HD is given by the likelihood under HD to that under HW and can be converted to a posterior probability that HD is true, given a prior probability. The LR and posterior probability for HW were also estimated. All the information related to the archaeological context, before any analysis was made, we considered as information that can inform the prior probability. See the Supplementary Information for further details.

2.2 Archaeogenetics

The methods undertaken for the archaeogenetic analyses are described in Pires et al. 2017 (Pires et al., 2017b). What follows is a brief summary.

2.2.1 Sub-sampling and aDNA isolation

A total of 46 *Canis* remains were sub-sampled for aDNA analyses, namely the six Mesolithic *Canis* (samples LYEP3, LYEP68A, LYEP68B, LYEP74, LYEP75 and SEP002), two Palaeolithic samples (LYEP44 and LYEP46) and one Chalcolithic sample (LYEP27) whose zooarchaeological analysis was described above. For comparison purposes, the remains of an additional 37 archaeological dogs from other periods were sub-sampled and included in the genetical analyses (for details see Sup. Table S5). Sub-sampling of archaeological remains was performed in the aDNA-dedicated facilities at PALGENE - French National Platform of Palaeogenetics at the École Normale Supérieure de Lyon (France) and the Archaeological Research Laboratory of Stockholm University (Sweden) following appropriate protocols to avoid contamination.

Bone or tooth powder (100–200 mg) was digested overnight with proteinase K (20 mg/mL) using specific protocols (Pires et al., 2017b). Following sample concentration with Amicon columns (Millipore-Amicon Ultra-4 30k Da), aDNA was extracted with the commercial QIAquick PCR Purification Kit from Qiagen. Ancient DNA was recovered following the manufacturer protocol in a final volume of 100μL. Two negative extraction controls were included in every batch of six samples as well as a sample for cross-contamination detection. Duplicates of aDNA extracts were obtained independently from each specimen.

2.2.2 Mitochondrial DNA amplification

Primer pairs DL1/DL3 and DL7/DL2 (Leonard et al., 2002) (primer sequence in Sup. Table S6) were used in independent amplification reactions to obtain a 181 base pair fragment from two overlapping *D-loop* fragments of 187 and 108 base pairs (bp), respectively, between nucleotide positions 15,495 and 15,676 in the dog reference sequence (EU789784). This fragment allows to discriminate between dog mtDNA haplogroups found in extant dogs (i.e., Hgs A, B, C and D).

Polymerase chain reactions (PCR) were carried out in a total volume of 25μL as previously described (Pires et al., 2017b). Primer sequences included multiplex identifiers for libraries (MIDs), i.e., 10 nucleotide sequence tags for multiplexing in emulsion PCR during 454-sequencing (Roche). Negative controls were systematically used to monitor possible contaminations and all extraction blanks were subject to PCR amplification. PCR products for the aDNA extracts duplicates were electrophoresed in agarose gels and purified for sequencing using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's recommendations.

2.2.3 Mitochondrial DNA sequencing

The 454-GS Junior technology (Roche) was used to sequence equimolar mixtures of pooled amplification products following the manufacturer's protocols. A bioinformatics pipeline on the GALAXY platform (Afgan et al., 2016) was used to demultiplex raw data from the sequencing in order to obtain one file for each PCR product using primer and MID information. Sequences' damage patterns were assessed with PhyloNet software (Helgason et al., 2007) to infer the ancient status of the DNA obtained (see Sup. Fig. S1). Reads were aligned using Muscle (Edgar, 2004). Sixty per cent consensus sequences were then generated for each individual from independent amplifications (ranging from 1-4

independent PCR replicas with an average of 2) using the Seaview software (Galtier et al., 1996). Partial mtDNA sequences of ancient *Canis* remains were deposited in GenBank, namely for the six Mesolithic dogs, two Palaeolithic wolves, and one Chalcolithic wolf; as well as for the additional 37 dog remains from other periods, namely Neolithic, Chalcolithic, Roman and Late Antiquity (see Sup. Table S5 for GenBank accession numbers).

2.2.4 Phylogenetic and statistical analyses

We investigated relationships between haplogroups and haplotypes. We first assigned each sequence to a major dog haplogroup (A, B, C or D) by constructing a Bayesian phylogenetic tree with comparison with well-defined dog matrilines retrieved from GenBank (Duleba et al., 2015). Sequences from modern Iberian native dog breeds (Pang et al., 2009; Pires et al., 2006) were also included. The software MrBayes v3.2.6 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) was used with the best-fitting evolutionary model GTR + I + gamma. The shape parameter of the gamma distribution was 0.634 and the proportion of invariable sites (I) was 0.521. We used 10,000,000 generations, sampling every 1,000[th] generation, and default settings for the remaining options. Convergence of the Monte Carlo Markov Chain and burn-in were determined through the analysis of the generations versus the log probability plot using the trace analysis tool TRACER v1.6. (Rambaut A. et al., 2014). The initial burn-in step discarded 20% of the sampled trees. This allowed for haplogroup assignment of all the Mesolithic dog mtDNA partial sequences with high confidence (Sup. Fig. S2).

Using the NETWORK v5.0.0.0 (Fluxus Technology Ltd, 2004–2016) software we then constructed Median-Joining (MJ) networks (Bandelt et al., 1999). Nucleotide (nt) substitutions weighted 10 for transitions and 30 for transversions. The identification of

dog haplogroups was further confirmed through comparison with reference sequence data of extant Iberian dogs and wolves trimmed to the 181 bp mtDNA fragment. These reference data comprise 23 haplotypes from extant Iberian dogs of known breed and village dogs (mongrels) (see Fig.S2 for the included reference sequences). An alignment of all these sequences/haplotypes is available from

JAS_SequenceAlignment_TheCuriousCaseoftheMesolithicIberianDogs_AEPiresetal (fasta file).

GenAlEx 6.501 software (Peakall and Smouse, 2006) was used to perform analysis of molecular variance (AMOVA) with genetic differentiation measured by pairwise PhiPT values. P-values were calculated based on 9,999 permutations.

3. Results and Discussion

3.1 The Mesolithic *Canis* remains identified as dogs

We studied six Mesolithic *Canis* (Fig. 2) dated by AMS ^{14}C to 7,903-7,570 years cal BP from the Muge shell-middens (n = 4), Sado shell-middens (n = 1) and Vale Boi in Algarve (n = 1). The four older samples consisted of isolated and fragmented remains while the two most recent samples were almost complete skeletons, most certainly associated with a new human concern towards dogs. Five Mesolithic *Canis* were measured (Sup. Table S1 and Figures 2-3). Their domesticated status was statistically determined based on their reduced size, archaeological context and diet. All samples are fully characterized in Tables 1-4.
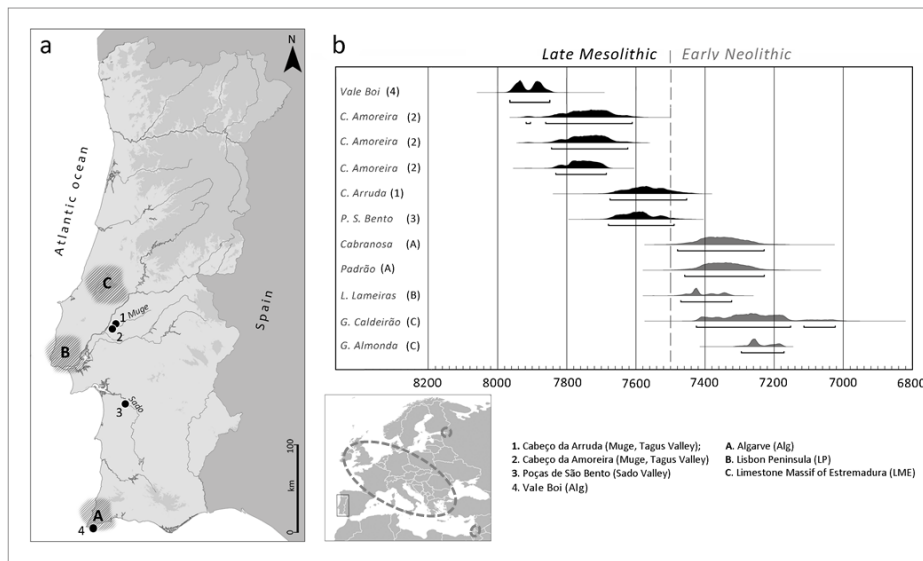
Fig. 2 – Early evidence for the presence of dogs in the Mesolithic of western Iberia: **(a)** Location of Mesolithic sites with *Canis* remains (1-4) and of the earliest Neolithic evidences in western Iberia (A-C); **(b)** Radiocarbon dates (cal BP, 95% confidence) of six *Canis* remains (bones and teeth) identified as dogs (in black) and for which mtDNA was analysed, and of non-canid species (in grey) recovered from Portuguese Neolithic sites. The vertical dashed grey line corresponds to the earliest Neolithic presence recorded in Portugal, at 7,500 years cal BP. The map of Europe shows the distribution of previous ancient *Canis* samples dated to 14,700 to 3,090 years BP as in (Frantz et al., 2016) - dashed line circles (Central Europe, plus five other samples: four from Estonia and one from Israel). Portugal is included in a rectangle.

a

b

c
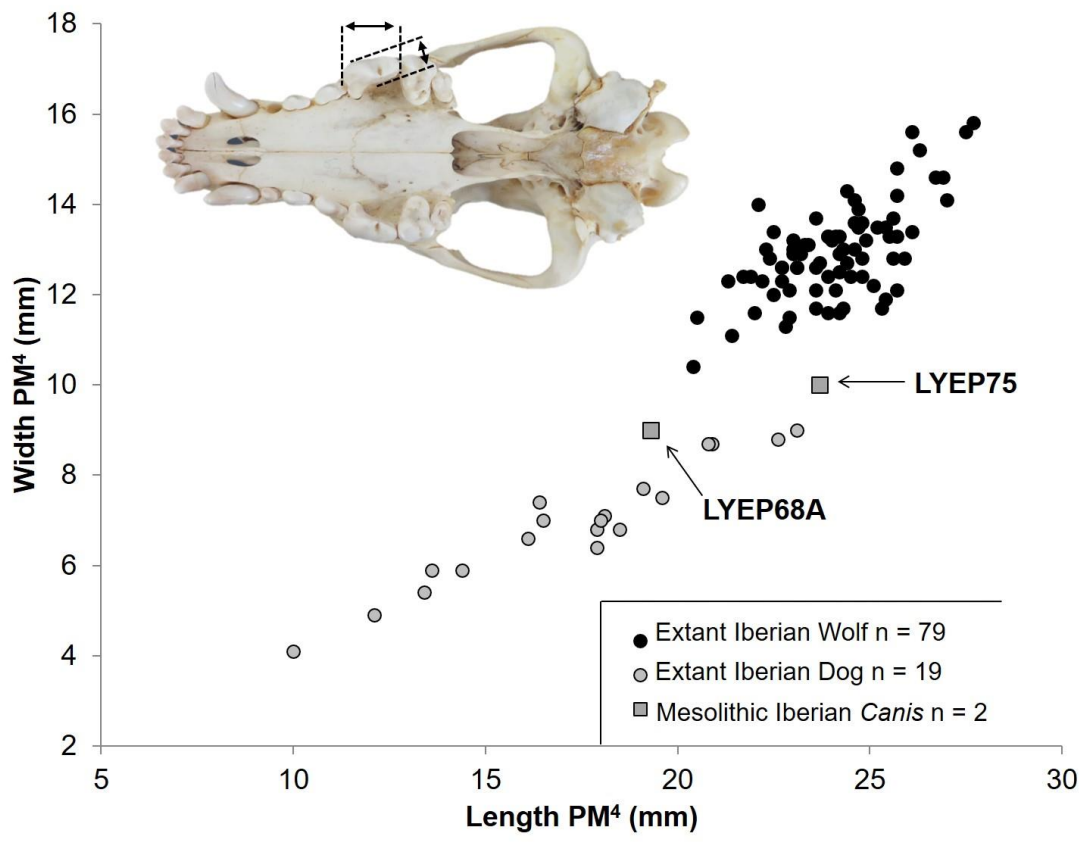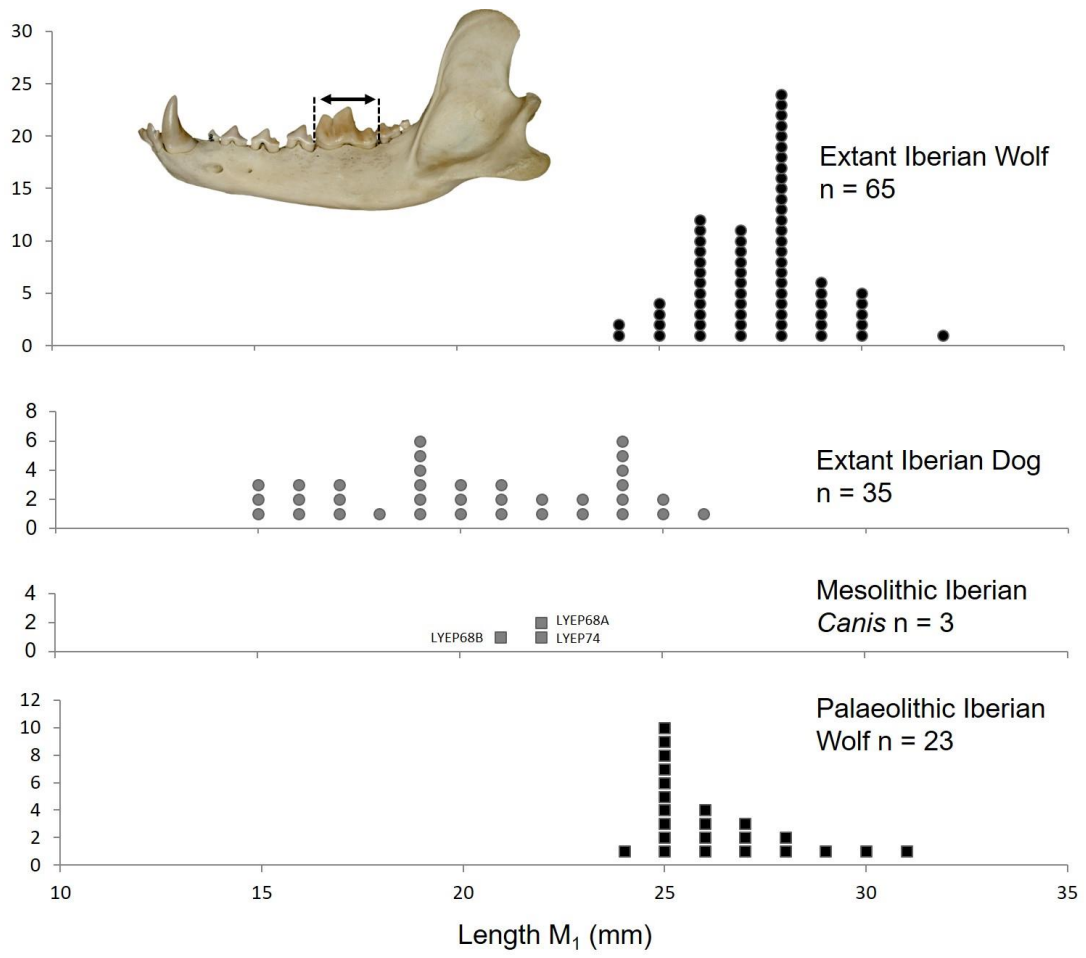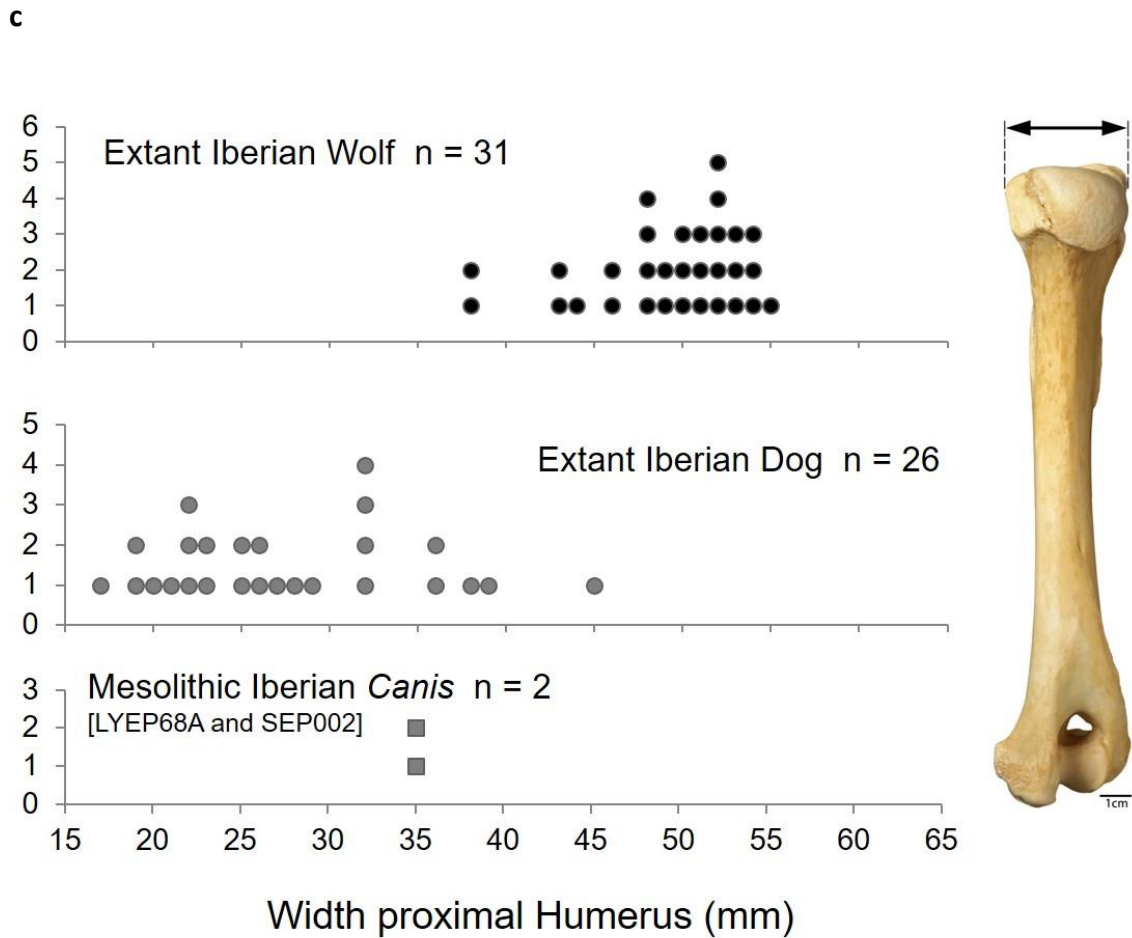
Fig. 3 - Domesticated status of Mesolithic *Canis*. Measurements of Mesolithic *Canis* remains (grey squares) compared to reference specimens of extant Iberian dogs (grey circles), and wolves (black circles) and Iberian Palaeolithic (Glacial and Interglacial periods) wolves (black squares): (**a**) breadth *versus* length measurements for the upper 4th pre-molar tooth (PM^4); (**b**) length of the lower 1st molar tooth (M$_1$); (**c**) width proximal humerus.

Using a non-parametric Kruskall-Wallis test we found a statistically significant difference in the length of the lower first molar (M$_1$) between extant, Upper Palaeolithic, and Middle Palaeolithic wolves from the reference osteometric database χ^2 ((2, N = 88) = 18.97, p < .001). After applying the Bonferroni correction, subsequent *post-hoc* pairwise comparisons using Mann-Whitney tests detected significant differences between: i) extant wolves and Middle Palaeolithic wolves and ii) Upper Palaeolithic wolves and Middle Palaeolithic wolves (Table 1). The magnitudes of these differences were large.

Table 1– *Post-hoc* pairwise comparisons of the M_1 tooth length in extant, Upper Palaeolithic and Middle Palaeolithic wolves. Measurements are given in millimeters.

Pairwise comparison	N	Mean	SD	Median	Range	Max.	Min.	Mann-Whitney U	Sig	Effect Size
Extant *vs* Upper Palaeolithic	65	27.40	1.47	27.50	7.70	31.50	23.80	248.5	.689	-
	7	27.83	1.81	27.50	6.00	31.00	25.00			
Extant *vs* Middle Palaeolithic	65	27.40	1.47	27.50	7.70	31.50	23.80	159.5	<.001	.48
	16	25.58	1.39	25.40	6.10	29.90	23.80			
Upper Palaeolithic *vs* Middle Palaeolithic	7	27.83	1.81	27.50	6.00	31.00	25.00	15.5	.007	.56
	16	25.58	1.39	25.40	6.10	29.90	23.80			
Extant *vs* Palaeolithic	65	27.40	1.47	27.50	7.70	31.50	23.80	408.0	.001	.34
	23	26.30	1.83	25.60	7.20	31.00	23.80			

The samples of extant and Upper Palaeolithic Iberian wolves presented non-significantly different M_1 lengths suggesting that few changes have occurred since then. One must bear in mind though, that the Upper Palaeolithic sample presents a hiatus of several millennia thus preventing reliable inferences. Also, the sample of Upper Palaeolithic wolves is very small and is certainly not entirely representative of this population - potentially leading to unreliable comparisons with the Mesolithic *Canis* specimens. As a result, for comparison with the Mesolithic *Canis* specimens, and as a more conservative approach, we intentionally increased the variability of the Palaeolithic wolves by pooling the two sets of samples (Middle and Upper Palaeolithic) into one group to assess if any difference between them is detected. By using this pooled sample, the comparison with the Mesolithic specimens becomes more conservative because we used the new larger interval as reference. Therefore, the Mesolithic *Canis* specimens were compared with extant and Palaeolithic Iberian wolves. The M_1 length values of the Mesolithic *Canis* were 20.5 mm (Cabeço da Amoreira, LYEP68B), 21.6 mm (Cabeço da Arruda, LYEP68A) and 21.7 mm (Poças de São Bento, LYEP74). Therefore, all values are smaller and outside the range recorded for both extant and Paleolithic Iberian wolf samples. We then assessed how different the Mesolithic *Canis* are from extant dogs. Prior to this, we had to establish if dogs can be reliably distinguished from wolves based on their dentitions and proximal humeral breadth. The Mann-Whitney test detected a significant difference (α = .01) with large effect sizes in M_1 length, PM^4 length and breadth, and breadth of the proximal humerus between extant dogs and extant wolves (Table 2). A tentative comparison of Mesolithic *Canis* specimens' measurements can be done by using the range of these variables, although with different resolutions according to each measurement because some overlap was detected for the PM^4 and the humerus

while no such overlapping was found for the M_1. Therefore, the latter seems to provide a better discrimination between dogs from wolves.

The values for Mesolithic *Canis* M_1 length range between 20.5 mm and 21.7 mm. Therefore, they are inside the range of our sample of extant dogs (Fig. 3) and, as seen above, well outside the range of our sample of extant Iberian wolves (Table 2).

The values for Mesolithic *Canis* PM^4 lengths were 19.3 mm (Cabeço da Arruda, LYEP68A) and 23.7 mm (Cabeço da Amoreira, LYEP75). The former is inside the range of our sample of extant dogs but the latter is more difficult to interpret. The same scenario is found for the Mesolithic *Canis* PM^4 breadths which were 9.0 mm (Cabeço da Arruda, LYEP68A) and 10.0 mm (Cabeço da Amoreira, LYEP75). The latter borderlines the range for both our samples of extant dogs and extant wolves (see Fig. 2a).

The breadths of the Mesolithic *Canis* proximal humeri were 35.0 mm (Cabeço da Arruda, LYEP68A) and 35.4 mm (Cabeço da Amoreira, SEP002). These values are well inside the range of our extant dogs and outside that of our extant wolves.

We also looked for significant differences between the mean values of M_1 length of extant dogs and Palaeolithic wolves. Extant dogs are among the most diverse mammals (Chase et al., 2002) and their descriptive statistics are expected to be significantly different from any group of wolves, regardless of their chronology. Indeed, the mean M_1 length of our sample of extant dog, which includes both small and large breeds, was significantly smaller than the mean of our sample of Palaeolithic wolves; also, the standard deviation and range of both sets of samples were very different (Table 2). Furthermore, there is little overlap between the two sets of samples.

Table 2 – Descriptive and inferential statistics of standard osteological and dental measurements (mm) between extant dogs and wolves and Palaeolithic wolves.

Pairwise comparison	N	Mean	SD	Median	Range	Max.	Min.	Statistic	Sig	Effect Size
Extant Dogs M_1 length *vs*	35	20.17	3.35	20.10	11.40	26.00	14.60	8.240*	<.001	1.74
Extant Wolves M_1 length	65	27.40	1.47	27.50	7.70	31.50	23.80			
Extant Dogs PM^4 length *vs*	19	17.32	3.49	17.90	13.10	23.10	10.00	21.350*	<.001	2.00
Extant Wolves PM^4 length	79	24.07	1.55	24.10	7.30	27.70	20.40			
Extant Dogs PM^4 breadth *vs*	19	6.93	1.34	7.00	4.90	9.00	4.10	12.151*	<.001	2.29
Extant Wolves PM^4 breadth	79	12.95	1.04	12.90	5.40	15.80	10.40			
Extant Dogs Proximal Humerus Breadth *vs*	26	27.57	7.27	26.05	28.40	45.00	16.60	13.266*	<.001	1.75
Extant Wolves Proximal Humerus Breadth	31	49.20	4.41	50.40	17.50	55.10	37.60			
Extant Dogs M_1 length *vs*	35	20.17	3.35	20.10	11.40	26.00	14.60	33.000**	<.001	1.11
Palaeolithic Wolves M_1 length	23	26.26	1.83	25.60	7.20	31.00	23.80			

* t-test: **Mann-Whitney test

This provides further support to our claim that the Iberian Mesolithic *Canis* presented in this paper are indeed dogs, since even an extremely diversified sample of extant dogs such as ours is different from both extant and ancient wolves and fails to overlap them. It is known that linear measurements fail to express all the variability in dogs and wolves, making it difficult to separate completely these two groups. Three dimensional geometric morphometrics of canid craniums provide a more accurate distinction for mandibles (Drake et al., 2017, 2015). Unfortunately, we have no complete skulls and LYEP68A is deformed.

Despite the limited size of our Mesolithic *Canis* and limitations of the caliper measurements, the comparison of standard measurements with known populations of Iberian wolves and dogs shows that the M_1, PM^4, and humeral measurements of the Mesolithic *Canis* are outside the range of extant or ancient wolves but within, or in some cases borderlining the range of extant Iberian dogs (Fig. 2; Tables 1 and 2). Therefore, we consider Mesolithic Iberian *Canis* remains to have belonged to dogs rather than wolves.

Inferences based merely on osteometry do not allow for absolute conclusions in every case. However, they show clear trends which, linked to other data such as dietary isotopes and burial contexts (see below), provide support for the identification of the Mesolithic specimens of *Canis* as dogs.

3.1.1 Isotopic data reveal that the diet of some Mesolithic *Canis* included a high percentage of aquatic food

The domesticated status of the Mesolithic *Canis* remains is further supported by archaeological context and isotopic $\delta^{13}C$ results. These indicate a high percentage of

aquatic protein in their diet (Table 3) – estimated through the equation established by Ambrose (1993) (Ambrose, 1993), suggesting co-habitation and diet-sharing with humans. In Mesolithic times, the Tagus estuary was larger with a stronger marine influence upstream where the shell midden sites are currently located (Vis et al., 2008).

Table 3 – Radiocarbon dates (cal BC and cal BP, 95% confidence limits) of specimens (bone collagen) recovered from Late Mesolithic (dark grey) and Early Neolithic (light grey) archaeological sites in Portugal. SD (Standard Deviation); ΔR (Marine Reservoir Offset); Mdn (Median). Radiocarbon dates were calibrated using OxCal v. 4.2 (Ramsey, 2009) with IntCal13 and Marine13 Curves (Reimer et al., 2013). Different ΔR values were used: 140±40 ^{14}C (Cabeço da Arruda and Cabeço da Amoreira shell-middens, Muge, Tagus Valley) (Martins et al., 2008), -100±155 ^{14}C (Poças de São Bento shell-midden, Sado Valley) and 95±15 ^{14}C (Cabranosa and Padrão, Algarve region) (Monge Soares et al., 2016). The proportion of aquatic protein in the diet was considered in the calibration of samples of *Canis* bone (Ambrose, 1993). * Value determined for tooth enamel sample (not quantifiable for diet determination purposes). See Fig. 2 for details on the geographical location of the archaeological sites and region codes.

Region	Archaeological site	Cultural Period	Sample/Species name	Common name	Lab. Reference	Age BP	SD	δ^{13}C‰	ΔR	Aquatic % ±10	cal BCE	Mdn	cal BP	Mdn
Tagus Valley	Muge (Cabeço da Arruda), Salvaterra de Magos	Late Mesolithic	*LYEP68A*		Beta-152956	7070	40	-14,4	140±40	70%	5730-5500	5620	7680-7450	7570
Sado Valley	Poças S. Bento, Alcácer do Sal	Late Mesolithic	*LYEP74*		OxA-26094	6866	33	-17,9	-100±155	26%	5735-5535	5645	7680-7485	7595
Tagus Valley	Muge (Cabeço Amoreira), Salvaterra de Magos	Late Mesolithic	*LYEP68B*		OxA-24571	7015	40	-18,14	140±40	23%	5965-5660	5785	7915-7605	7735
Tagus Valley	Muge (Cabeço Amoreira), Salvaterra de Magos	Late Mesolithic	*LYEP75*		WK-36713	6971	33	-19,05	140±40	12%	5895-5675	5790	7845-7625	7735
Tagus Valley	Muge (Cabeço Amoreira), Salvaterra de Magos	Late Mesolithic	*SEP002*		Beta-448544	6930	30	-20,5	-	-	5885-5735	5805	7835-7685	7755
Algarve	Vale Boi (Algarve)	Late Mesolithic	*LYEP3*		Beta -459997	7080	30	-10,9 *	-	-	6016-5899	5954	7965-7848	7903
Algarve	Cabranosa	Early Neolithic	*Mytilus* sp.	mussel	Sac-1321	6930	65	-	95±15	-	5530-5280	5405	7475-7225	7355

Algarve	Padrão	Early Neolithic	*R. decussatus*	clam	ICEN-873	6920	60	-	95±15	-	5510-5275	5395	7460-7225	7345
Lisbon Peninsula	Lapiás das Lameiras	Early Neolithic	*O. aries*	sheep	OxA-29109	6497	34	-	-	-	5525-5370	5470	7475-7320	7420
Limestone Massif of Estremadura	Caldeirão cave	Early Neolithic	*O. aries*	sheep	OxA-1035	6330	80	-	-	-	5480-5075	5310	7425-7020	7260
Limestone Massif of Estremadura	Almonda cave	Early Neolithic	*H. sapiens*	human	MAMS-18262	6319	22	-19,9	-	-	5350-5220	5305	7295-7170	7250

As described above, we studied four chronologically older isolated dog remains and two more recent and almost complete skeletons whose diets included a high percentage of aquatic food (70% and 26%, estimated from isotopic data, Table 3). For human remains from the Mesolithic shell-middens in the Tagus valley this percentage is frequently higher than 50% (Stjerna, 2016; Umbelino, 2006). The two dog skeletons were well preserved which probably reflects the extra care provided by their human owners during burial. The older isolated remains were poorly preserved and may represent animals that were not intentionally buried. The distinct diet determined for the later Mesolithic dog skeletons, rich in aquatic/marine resources, reinforces the hypothesis of special treatment given to these two animals.

Aquatic diet alone may not be a strong argument for their domestic status. Grey wolves, mainly those from coastal areas with access to a marine seasonal food resource with high caloric content (e.g., spawning salmon) may rely on a marine diet when terrestrial ungulates are in short supply (Paquet and Carbyn, 2003) or not (e.g. (Darimont et al., 2008) for the extant coastal wolves of British Columbia (Canada). Another wolf species, the extinct Ezo wolf (*Canis lupus hattai*) in Japan, also had a marine diet (Matsubayashi et al., 2017). The feeding habits of extant European wolves have been characterized. Based on a review of extant grey wolf diet (177 studies), fish are supplementary prey for wolves in Europe (Northern Spain), in present times (Newsome et al., 2016). In an earlier study of Iberian wolves from Spain, 251 stomach contents were analysed and the authors concluded that fish only constituted a small part of the diet of wolves (Cuesta et al., 1991). The Iberian wolf diet, in prehistoric times, is unknown, but with availability of wild terrestrial prey in the Muge area – such as red deer, roe deer, wild boar, auroch, rabbit, hare (Detry, 2007), fish consumption could be supplementary as well. Only a direct isotopic analysis of samples of archaeological bone would provide insights into this

subject. Unfortunately, these data are not available at the moment. In the three prehistoric wolves analysed, one was dated with the Uranium–thorium method which does not provide information regarding $\delta^{13}C$ content, another sample could not be radiocarbon dated due to lack of bone collagen and for the other (Chalcolithic) we only have an indirect dating. But, even in an alternative scenario where prehistoric Iberian wolves have consumed large amounts of fish, the osteometry and archaeological context also constitute strong evidences that the Mesolithic *Canis* remains are indeed dogs (see below posterior probabilities estimation).

3.1.2 Depositional context

The Mesolithic *Canis* remains studied here are all derived from archaeological contexts, i.e. from sites and layers and sites where evidence of past human activities is present. The Pleistocene *Canis* were excavated in geological contexts presenting no evidence of human activities. The only *Canis*, supposed to be a wolf and coming from an archaeological site is represented by a single tooth and dated to the Chalcolithic.

Being a domestic species, dogs are naturally associated with humans and therefore to archaeological sites. The four older Mesolithic *Canis* are fragments and the two more recent ones are represented by complete skeletons. This suggests that these more recent ones had been buried as part of some kind of ritual. The fact that a complete skeleton is preserved across time implies that its rapid sedimentation occurred by covering the carcass – an event that was probably done intentionally by people. The specimen LYEP74, discovered in 2011 (Fig. 1C), shows a constricted body intentionally buried and deposited with care, in the same way as humans. The specimens represented by scattered fragments only, were probably not subjected to a careful burial and therefore were not preserved in their entirety.

Mesolithic shell middens in the European Atlantic façade often show human and dog burials in close vicinity (Larsson, 1990), as well as in the Natufian period, in the Middle East (Davis and Valla, 1978), suggesting that hunter-gatherers had a strong affection for dogs.

3.1.3 Dog/Wolf Bayesian statistical assignment

Finally, we statistically classified our samples as dog or wolf taking into account all the archaeological, osteometric, direct dating and isotopic content information available (see Table 4 and Sup. Material for more details).

Table 4 – Description and species statistically-based assignment of the Palaeolithic to Chalcolithic samples of this study, considering archaeological data. Posterior probabilities for the hypotheses of being a dog (HD) or wolf (HW) were estimated after calculating the likelihoods for each hypothesis (see Supplementary Information for details). For details on calibrated dates of Mesolithic *Canis* samples see Table 3.

Sample (lab code)	Archaeological site	Cultural Period	Skeletal element recovered	Posteriors HD	Posteriors HW	Species statistically-based assignment
LYEP68A	Muge (Cabeço da Arruda), Salvaterra de Magos	Late Mesolithic	Complete skeleton	0.999	~0	**Dog**
LYEP74	Poças S. Bento, Alcácer do Sal	Late Mesolithic	Complete skeleton	0.999	~0	**Dog**
LYEP68B	Muge (Cabeço Amoreira), Salvaterra de Magos	Late Mesolithic	Mandible (with P2-M2)	0.999	~0	**Dog**
LYEP75	Muge (Cabeço Amoreira), Salvaterra de Magos	Late Mesolithic	Maxillary (with dP^4, P^4, M^1)	0.622	0.006	**Dog**
SEP002	Muge (Cabeço Amoreira), Salvaterra de Magos	Late Mesolithic	Proximal humerus	0.999	~0	**Dog**
LYEP3	Vale Boi (Algarve)	Late Mesolithic	Tooth	0.320	0.020	**Likely Dog**
LYEP27	Penedo Lexim, Mafra	Chalcolithic	Lower molar 1	~0	0.999	**Wolf**
LYEP44	João Ramos cave, Alcobaça	Upper Palaeolithic	Mandible	0.020	0.320	**Likely Wolf**
LYEP46	Furninha cave, Peniche	Middle Palaeolithic	Tooth root	~0	0.998	**Wolf**

Four of our Mesolithic samples were classified as dogs with a posterior probability of 99%. Samples LYP75 and LYP3 have lower values (62% and 32%), however they were also classified as dog or likely dog due to their posterior probabilities for the alternative hypothesis HW (wolf) being very low (0.6% and 2%, respectively). We classified the Chalcolithic sample LYP27 and Palaeolithic one LYP46 as wolves with a posterior probability of 99%. Sample LYP44 was also classified as likely wolf but with a low posterior probability (32%), although higher than the one for HD (dog). There are few data available for samples LYP3 and LYP44 and the majority of their posterior values (for HD and HW, respectively) are given by prior information i.e. archaeological context, which explains their low posterior probability values.

3.2 Dog matriline A is present at high frequency in Iberia since the Mesolithic

This is the first study focusing on the genetic analysis of early dog specimens in Iberia. Regarding mtDNA sequence variability, the four dog remains and one of the Mesolithic skeletons belong to dog mt-haplogroup A (HgA), and the most recent skeleton to dog mt-haplogroup C (HgC) (Table 5). A phylogenetic tree with support values demonstrating that clades can effectively be differentiated with such a small fragment is presented in Sup. Fig. S2.

Table 5 – Description of the Palaeolithic to Mesolithic samples studied and a Chalcolithic sample, considering genetic data. For details on calibrated dates of Mesolithic *Canis* samples see Table 3. Data for the remaining archaeological dog samples are presented in Sup. Table S5.

Sample (lab code)	Species	mtDNA sequence (base pairs)	454 sequencing coverage	GenBank Accession number	Dog mtDNA Haplogroup (Hg)
LYEP68A	**Dog** (*Canis l. familiaris*)	110	126	KY014676	Dog Hg C
LYEP74	**Dog** (*Canis l. familiaris*)	181	167	KY014682	Dog Hg A
LYEP68B	**Dog** (*Canis l. familiaris*)	181	205	KY014677	Dog Hg A
LYEP75	**Dog** (*Canis l. familiaris*)	181	598	KY014683	Dog Hg A
SEP002	**Dog** (*Canis l. familiaris*)	138	38	KY014675	Dog Hg A
LYEP3	**Dog** (*Canis lupus familiaris*)	165	2369	KY014652	Dog Hg A
LYEP27	**Iberian Wolf** (*Canis lupus*)	129	794	KY014649	
LYEP44	**Iberian Wolf** (*Canis lupus*)	165	17	KY014650	
LYEP46	**Iberian Wolf** (*Canis lupus*)	165	34	KY014651	

Based on current data (Ollivier et al., 2018), the differentiation of the Iberian Mesolithic dogs within the European context is unexpected. Indeed, dog HgA haplotypes are present at a high frequency before the arrival of the Neolithic (five out of the six Mesolithic samples studied (83%)) and remained at high frequency (>50% in all samples), with an overall average of 69% across all ancient Iberian samples (Fig. 4). In a previous study (Frantz et al., 2016) the frequency of all HgA haplotypes found in Europe was lower than 9%, with only 5 haplogroup A haplotypes in 59 sequences, for the period 14,700 to 3,090 BP. As noted above, only one Iberian Mesolithic dog specimen carried a HgC haplotype, even though it was the most frequently observed haplogroup in ancient dogs from other parts of Europe before the Neolithic (Frantz et al., 2016).
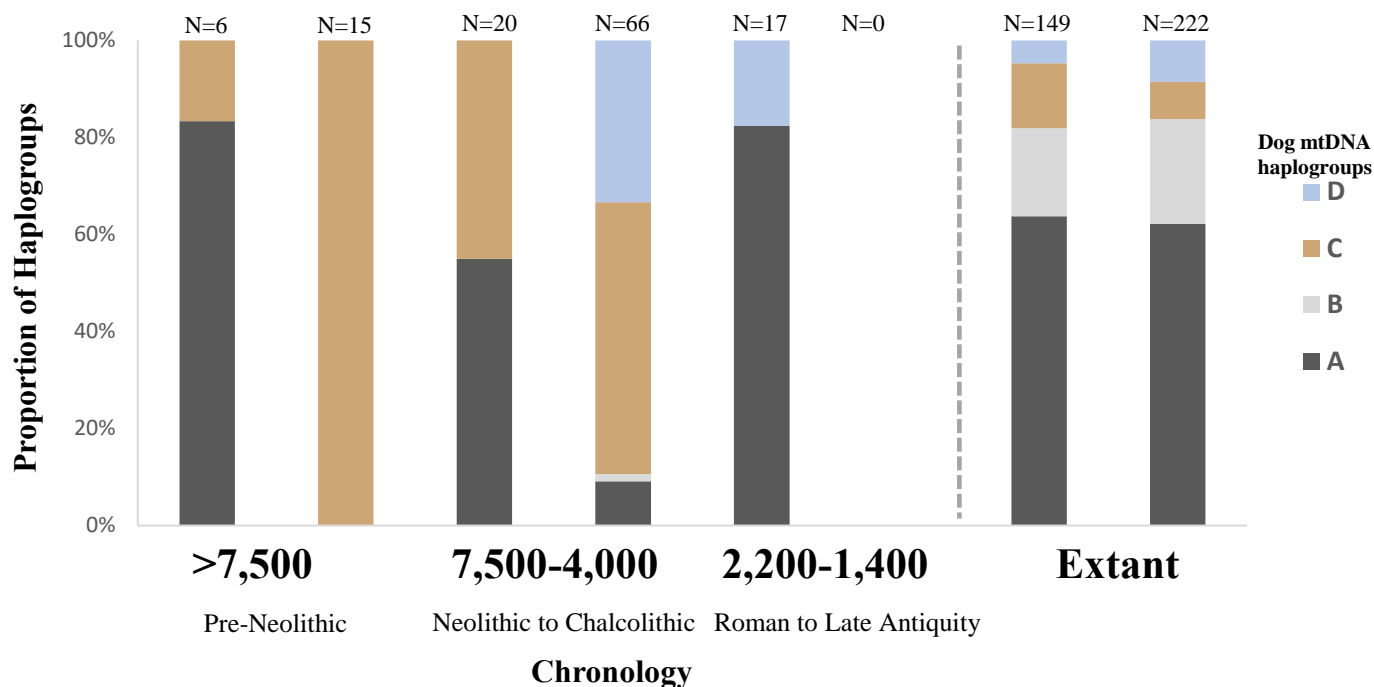
Fig. 4 – Frequency of the main dog mtDNA-haplogroups (A, B, C and D) across time in Iberia (left bars) and the rest of Europe - Western Northern fringe, Central Western and South-Eastern (right bars). Dates are in cal BP. A high frequency of HgA dogs can be detected continuously in Iberia since the Mesolithic.

Curiously, the haplogroup A haplotypes detected in Iberian Mesolithic dogs differ from that described for contemporary Middle East dog (Frantz et al., 2016) by 1-2 nucleotides, while the Mesolithic Iberian dog haplogroup C haplotype is shared by other contemporary dogs from Europe (Romania, Estonia, Germany and France) described in Franz et al. study (Frantz et al., 2016) (see Sup. Fig. S3), which reinforces the possibility of pre-Neolithic local processes for Iberian wolf domestication. Given the high frequency of mtDNA haplogroup A for Mesolithic Iberian dogs (our study), the contrast with other contemporary dogs from the northern/central western Europe, and the geographic distance from Near-Middle East Natufian dogs carrying haplogroup A (Ollivier et al.,

2018) and the consequent lack of gene flow, we cannot exclude a local/independent process of domestication (recruitment) for Iberian wolves (note: we do not claim that Iberia was a centre of wolf domestication). Additionally, no other empirical data or specific cultural behaviours exist in Mesolithic Iberia that could imply contacts with Middle East, as happened later during the Neolithic (Isern, Zilhão, Fort, & Ammerman, 2017). A possible contact with Middle East via a trans-Mediterranean western route through North Africa is only reported in the Neolithic, ~4,000 years before present (González-Fortes et al., 2019).

Wolf and dog haplogroups frequencies varied both across time (in Central Europe) (Pilot et al., 2010) and space (Iberia *versus* Central Europe) (Pilot et al., 2014, 2006). One could speculate that wolf populations (and consequently dogs) were genetically structured, both prior to and after domestication, with different frequencies of lineages in different populations for each species. However, demographic models are needed to further infer accurately admixture/migration events. Genetic data suggest that the increase in the frequency of the HgA in extant dogs appears to be recent (post-Neolithic) in central and northern regions of Europe but older in Iberia. Moreover, it does not necessarily require the arrival of wolves or HgA dogs from outside Europe. The presence of distinct maternal lineages suggests that the genetic diversity and differentiation of Iberian *Canis* was already high relatively to their European counterparts, probably due to geographical isolation, but additional data on past Iberian and European wolf populations are needed.

3.3 A close genetic affinity between Mesolithic dogs and Palaeolithic wolves in Iberia

Mesolithic dog haplotypes segregate within HgA (two haplotypes, one of them shared with a Palaeolithic wolf) and HgC (one haplotype shared with another Palaeolithic wolf) (Fig. 5).
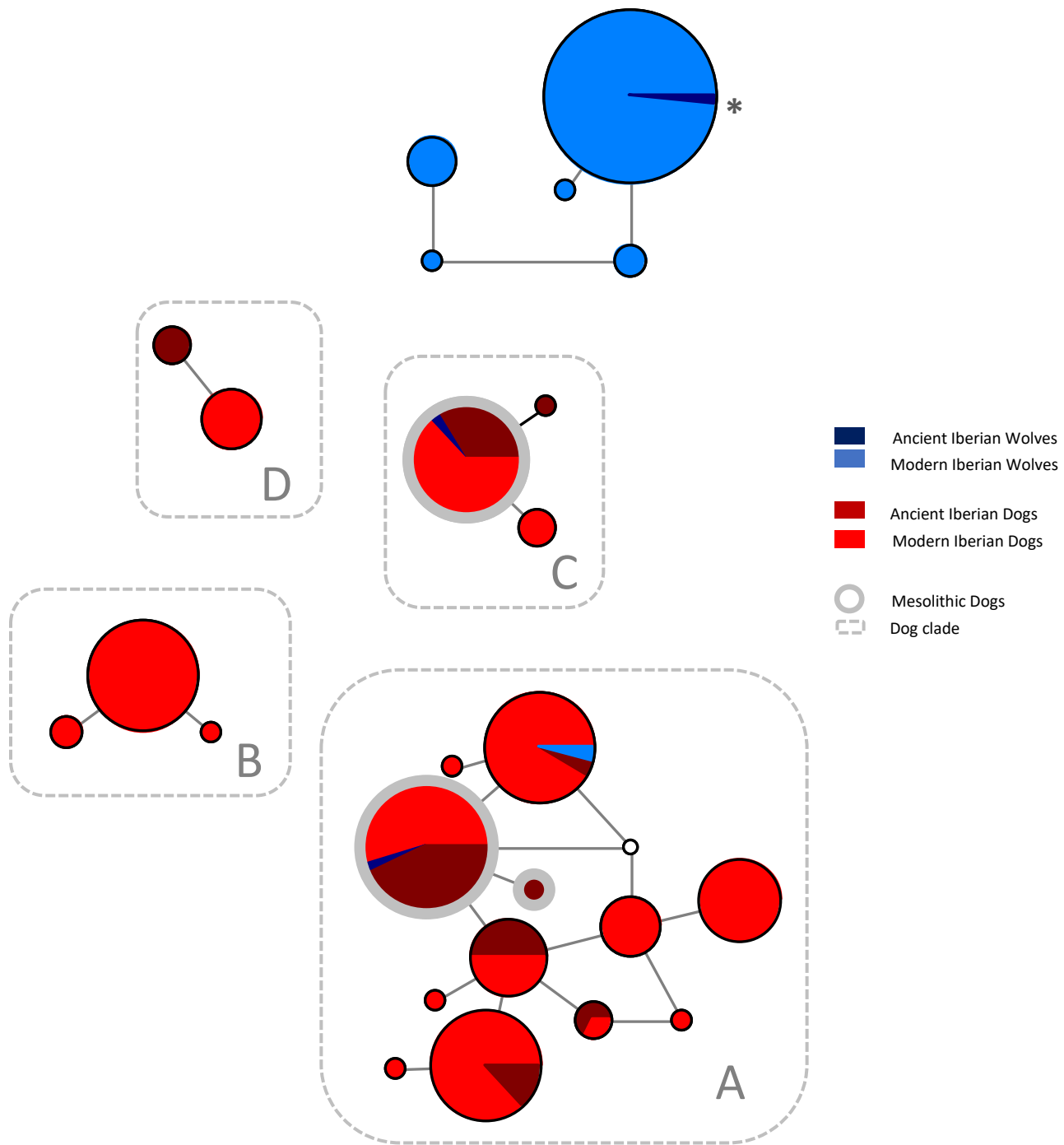
Fig. 5 – Median-Joining networks displaying 181 base pair long mtDNA-haplotypes found in ancient and extant Iberian *Canis* samples. Dog mtDNA haplogroups: A, B, C and D. Ancient Iberian dogs include dogs dated from Mesolithic to Late Antiquity times. In this network the links between haplogroups were removed because with such a small mtDNA fragment clades differentiation is recovered but not the topology between them, as when a larger fragment is used as in (Pires et al., 2006) for genetic data from extant Iberian *Canis lupus familiaris*. A MJ network

with links and mutated positions between haplotypes is available in Sup. Fig. S4. The Chalcolithic wolf is indicated with an asterisk. The white circle indicates a median vector.

Although partial sequence fragments with a low coverage were recovered from Iberian Palaeolithic wolf samples (Table 5), these haplotypes are shared by Mesolithic and extant Iberian dogs, probably due to incomplete lineage sorting, but are different from those of extant Iberian wolves. This contrasts with the Chalcolithic wolf sample which shares its haplotype with extant Iberian wolves in a genetically distinct wolf cluster (Fig. 5).

The genetic differentiation between the Palaeolithic Iberian wolves and their extant counterparts is confirmed by the high PhiPT of 0.915 (*p*-value = 0.001, Table 6). It is interesting that they are genetically much closer to Mesolithic dogs (negative PhiPT estimated as 0, non-significant).

This difference between Palaeolithic and Chalcolithic/extant wolves suggests that there may have been a change in the mtDNA composition of wolves in Iberia, and that Mesolithic dogs kept the genetic signature of the ancient diversity of Iberian wolves, up to the present-day dogs. The strong genetic differentiation between present-day wolves and dogs in Iberia (0.490, *p*-value = 0) is also reflected by Y-chromosome data (Pires et al., 2017a), and reported for other *Canis* populations as well (Thalmann et al., 2013).

Table 6 – Genetic population differentiation measured by pairwise PhiPT values and its statistical significance. PhiPT values are indicated below the diagonal and in bold. Probability, *P* (rand >= data) based on 9,999 permutations is shown above diagonal and in italics.

Dogs		Wolves		
Mesolithic	Modern	Modern	Palaeolithic	
-	*0.001*	*0.000*	*0.355*	Mesolithic Dogs
0.313	-	*0.000*	*0.002*	Modern Dogs
0.628	**0.490**	-	*0.001*	Modern Wolves
0.000	**0.771**	**0.915**	-	Palaeolithic Wolves

Only a few ancient Iberian wolf samples were analysed here, however the discontinuity observed between Palaeolithic wolves and their extant counterparts is interesting and warrants further investigation. It has been suggested that ancient wolves adapted to new ecological niches created by humans such as garbage dumps, possibly as a consequence of a specific fearless phenotype and small flight distance and thus began a process known as *self-domestication* (Coppinger and Coppinger, 2002; Morey and Jeger, 2015). The remaining wolves that survived untamed until today are the ones who would have maintained their distance from humans. The fact that the Chalcolithic Iberian wolf analyzed here exhibits the most common haplotype detected in extant Iberian wolves but rare in Palaeolithic wolves could suggest that to some extent the maternal genetic composition of the wolf populations in Iberia changed over time. This scenario is consistent with a chronological turnover of Eurasian wolf lineages previously detected by Freedman et al., (2014). They found that extant wolf lineages from putative domestication centers form a sister monophyletic haplogroup of

the dog haplogroup and therefore are not the direct lineal ancestors of dogs. In the Iberian wolf population, a long-term demographic bottleneck (which possibly started in the Neolithic) was identified. And signatures of genetic drift due to spatial isolation and diversifying selection inferred from the analysis of genome-wide SNPs (Pilot et al., 2014) were recognized. A more specialized Iberian wolf feeding behaviour in the past (an ecomorph) (as for the Late Pleistocene wolves from eastern Beringia (Leonard et al., 2007)) and/or changes in the ecological environment and evolutionary processes across time (see (Darimont et al., 2009; Pilot et al., 2006; Stronen et al., 2014) for ecological heterogeneity across space) could have contributed to the differences observed between ancient and extant Iberian wolf matrilines as already suggested by Pilot and collaborators (Pilot et al., 2010). Natural ecological factors have changed but more recent factors owing to human demographic expansion have been determinant to the observed genetic structure.

Our data also provide an interesting interpretation for a large canid specimen found in the Romanelli cave (sample PIC3) in Apulia, southern Italy (Sardella et al., 2014; Verginelli et al., 2005). This canid, dated to $9,670 \pm 40$ years BP, carries an HgA-haplotype (Verginelli et al., 2005) and a sequence that does not differ from our most frequent Iberian Mesolithic HgA-haplotype. Whether it was a dog or a wolf, it suggests that HgA was more frequent and geographically widespread in southern Europe than previously thought, both among wolves and earliest domesticated dog populations and well before the arrival of the Neolithic. If we assume this canid was a dog it would support the hypothesis that local independent wolf domestication events occurred.

Conclusion

The earliest known dog remains from the Iberian Peninsula were studied and we report a regional high frequency of mtDNA HgA in pre-Neolithic European dogs which contrasts with other contemporary European dog populations. Our study shows that East Asia and the Middle East may not have been the only sources for HgA-type dogs in some parts of Europe. Whereas previous data evidenced expansions of dog population from East Asia (out of Asia) to the West during the Neolithic which caused a turnover in the mitochondrial ancestry of European dogs and shaped the patterns of genetic differentiation in modern dogs (e.g. (Frantz et al., 2016; Pilot et al., 2015; Wang et al., 2016)); the observed high frequency of mtDNA HgA in pre-Neolithic Iberia probably reflects the spatial genetic structure of the ancestral population – the Palaeolithic wolf. Our results, although based on a limited sample size and a single genetic marker, emphasize that knowledge of both ancient wolves' and early dogs' genetic profiles from regions on the European periphery should improve our understanding of the evolution of the European dog.

References:

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., Grüning, B., Guerler, A., Hillman-Jackson, J., Von Kuster, G., Rasche, E., Soranzo, N., Turaga, N., Taylor, J., Nekrutenko, A., Goecks, J., 2016. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res. 44, W3–W10. doi:10.1093/nar/gkw343

Ambrose, S., 1993. Isotopic analysis of palaeodiets: methodological and interpretive considerations., in: Ed., M.K.S. (Ed.), Investigations of Ancient Human Tissue. Chemical Analyses in Anthropology. Langhorne: Gordon and Breach Science Publishers, pp. 59–130.

Antunes, M.T., Cabral, J.M.., Cardoso, J.L., Pais, J., Soares, A.M., 1989. Paleolítico médio e superior em Portugal: datas 14C, estado actual dos conhecimentos, síntese e discussão. Ciências da Terra 10, 127–138.

Arias, P., Diniz, M., Araújo, A., Armendariz, A., Teira, L., 2015. At the Edge of the Marshes: New Approaches to the Sado Valley Mesolithic (Southern Portugal)., in: Bicho, N., Detry, C., Price, T., Cunha, E. (Eds.), Muge 150th: The 150th Anniversary of the Discovery of Mesolithic Shellmiddens. Cambridge Scholars Publishing, Cambridge, pp. 301–319.

Arias, P., Diniz, M., Cubas, M., Duarte, C., Iriarte, E., Salzmann, C., Teichner, F., Teira, L., 2016. Looking for the traces of the last hunter–gatherers: Geophysical survey in the Mesolithic shell middens of the Sado valley (southern Portugal). Quat. Int. 435,Part B, 61–70. doi:10.1016/j.quaint.2016.02.016

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.

Bergmann, C., 1848. Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Größe. Vandenhoeck & Ruprecht, Göttingen.

Bicho, N., Cascalheira, J, J, M., 2012. On the (L)edge: the case of Vale Boi rockshelter (Algarve, Southern Portugal), in: Skeates, K.B. and R. (Ed.), Caves in Context. The Economical, Social, and Ritual Importance of Caves and Rockshelters. Oxbow Books, Oxford, Oxford, pp. 65–81.

Bicho, N., Manne, T., Marreiros, J., Cascalheira, J., Pereira, T., Tátá, F., Évora, M., Gonçalves, C., Infantini, L., 2013. The ecodynamics of the first modern humans in Southwestern Iberia: The case of Vale Boi, Portugal. Quat. Int. 318, 102–116. doi:10.1016/j.quaint.2013.06.029

Botigué, L.R., Song, S., Scheu, A., Gopalan, S., Pendleton, A.L., Oetjens, M., Taravella, A.M., Seregély, T., Zeeb-Lanz, A., Arbogast, R.-M., Bobo, D., Daly, K., Unterländer, M., Burger, J., Kidd, J.M., Veeramah, K.R., 2017. Ancient European dog genomes reveal continuity since the Early Neolithic. Nat. Commun. 8, 16082. doi:10.1038/ncomms16082

Cardoso, J., 1993. Contribuição para o conhecimento dos grandes mamíferos do Plistocénico Superior de Portugal (PhD thesis). Universidade Nova de Lisboa.

Chase, K., Carrier, D.R., Adler, F.R., Jarvik, T., Ostrander, E.A., Lorentzen, T.D., Lark, K.G., 2002. Genetic basis for systems of skeletal quantitative traits: Principal component analysis

of the canid skeleton. Proc. Natl. Acad. Sci. 99, 9930.

Clutton-Brock, J., 1999. A Natural History of Domesticated Mammals. Cambridge University Press.

Coppinger, R., Coppinger, L., 2002. Dogs : a new understanding of canine origin, behavior, and evolution. University of Chicago Press.

Cuesta, L., Barcena, F., Palacios, F., Reig, S., 1991. The trophic ecology of the Iberian Wolf (Canis lupus signatus Cabrera, 1907). A new analysis of stomach's data. Mammalia 55, 239–254. doi:https://doi.org/10.1515/mamm.1991.55.2.239

Cunha, E., Cardoso, F., Umbelino, C., 2003. Inferences about Mesolithic life style on the basis of anthropological data. The case of the Portuguese shell middens, in: Larsson, L. (Ed.), Mesolithic on the Move: Papers Presented at the Sixth International Conference on the Mesolithic in Europe. Oxbow Books, Oxford, pp. 184–188.

Darimont, C.T., Paquet, P.C., Reimchen, T.E., 2009. Landscape heterogeneity and marine subsidy generate extensive intrapopulation niche diversity in a large terrestrial vertebrate. J. Anim. Ecol. 78, 126–133. doi:10.1111/j.1365-2656.2008.01473.x

Darimont, C.T., Paquet, P.C., Reimchen, T.E., 2008. Spawning salmon disrupt trophic coupling between wolves and ungulate prey in coastal British Columbia. BMC Ecol. 8, 14. doi:10.1186/1472-6785-8-14

Darwin, C., 1885. The Variation of Animals and Plants under Domestication (Volume I), 2nd ed.

Davis, S.J.M., Simões, T., 2016. The velocity of Ovis in prehistoric times: the sheep bones from Early Neolithic Lameiras, Sintra, Portugal. Monogr. AAP 51–66.

Davis, S.J.M., Svensson, E.M., Albarella, U., Detry, C., Götherström, A., Pires, A.E., Ginja, C., 2012. Molecular and osteometric sexing of cattle metacarpals: A case study from 15th century AD Beja, Portugal. J. Archaeol. Sci. 39, 1445–1454.

Davis, S.J.M., Valla, F.R., 1978. Evidence for domestication of the dog 12,000 years ago in the Natufian of Israel. Nature 276, 608–610. doi:10.1038/276608a0

Detry, C., 2007. Paleoecologia e Paleoeconomia do Baixo Vale do Tejo – Fauna Terrestre dos Concheiros de Muge. Universidade de Salamanca.

Detry, C., Cardoso, J.L., 2010. On some remains of dog (Canis familiaris) from the Mesolithic shell-middens of Muge, Portugal. J. Archaeol. Sci. 37, 2762–2774. doi:10.1016/j.jas.2010.06.011

Drake, A.G., Coquerelle, M., Colombeau, G., 2015. 3D morphometric analysis of fossil canid skulls contradicts the suggested domestication of dogs during the late Paleolithic. Sci. Rep. 5, 8299. doi:10.1038/srep08299

Drake, A.G., Coquerelle, M., Kosintsev, P.A., Bachura, O.P., Sablin, M., Gusev, A. V., Fleming, L.S., Losey, R.J., 2017. Three-Dimensional Geometric Morphometric Analysis of Fossil Canid Mandibles and Skulls. Sci. Rep. 7, 9508. doi:10.1038/s41598-017-10232-1

Duleba, A., Skonieczna, K., Bogdanowicz, W., Malyarchuk, B., Grzybowski, T., 2015. Complete

mitochondrial genome database and standardized classification system for Canis lupus familiaris. Forensic Sci. Int. Genet. 19, 123–129. doi:10.1016/j.fsigen.2015.06.014

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. doi:10.1093/nar/gkh340

Fan, Z., Silva, P., Gronau, I., Wang, S., Armero, A.S., Schweizer, R.M., Ramirez, O., Pollinger, J., Galaverni, M., Ortega Del-Vecchyo, D., Du, L., Zhang, W., Zhang, Z., Xing, J., Vilà, C., Marques-Bonet, T., Godinho, R., Yue, B., Wayne, R.K., 2016. Worldwide patterns of genomic variation and admixture in gray wolves. Genome Res. 26, 163–73. doi:10.1101/gr.197517.115

Frantz, L., Mullin, V.E., Pionnier-Capitan, M., Lebrasseur, O., Ollivier, M., Perri, A., Linderholm, A., Mattiangeli, V., Teasdale, M.D., Dimopoulos, E.A., Tresset, A., Duffraisse, M., McCormick, F., Bartosiewicz, L., Gál, E., Nyerges, É.A., Sablin, M. V, Bréhard, S., Mashkour, M., Bălăşescu, A., Gillet, B., Hughes, S., Chassaing, O., Hitte, C., Vigne, J.-D., Dobney, K., Hänni, C., Bradley, D.G., Larson, G., 2016. Genomic and archaeological evidence suggest a dual origin of domestic dogs. Science 352, 1228–31. doi:10.1126/science.aaf3161

Galtier, N., Gouy, M., Gautier, C., 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Bioinformatics 12, 543–548. doi:10.1093/bioinformatics/12.6.543

González-Fortes, G., Tassi, F., Trucchi, E., Henneberger, K., Paijmans, J. L. A., Díez-del-Molino, D., … Hofreiter, M. (2019). A western route of prehistoric human migration from Africa into the Iberian Peninsula. *Proc. R. Soc. B*, *286*(1895). https://doi.org/https://doi.org/10.1098/rspb.2018.2288

Helgason, A., Pálsson, S., Lalueza-Fox, C., Ghosh, S., Sigurðardóttir, S., Baker, A., … Stefánsson, K. (2007). A Statistical Approach to Identify Ancient Template DNA. Journal of Molecular Evolution, 65(1), 92–102. https://doi.org/10.1007/s00239-006-0259-8

Habermehl, K.-H., 1975. Die Altersbestimmung bei Haus- und Labortieren. Parey, Berlin.

Horard-Herbin, M.-P., 2001. Dog management and use in the late Iron age: the evidence from the gallic site of Levroux (France), in: Proceedings of the First ICAZ Symposium on the History of the Domestic Dog. p. 115.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian Inference of Phylogeny and Its Impact on Evolutionary Biology. Science 294, 2310–2314. doi:10.1126/science.1065889

Isern, N., Zilhão, J., Fort, J., & Ammerman, A. J. (2017). Modeling the role of voyaging in the coastal spread of the Early Neolithic in the West Mediterranean. *Proceedings of the National Academy of Sciences*, *114*(5), 897–902. https://doi.org/10.1073/pnas.1613413114

Larsson, L., 1990. Dogs in fraction - symbols in action, in: Vermeersch, P., Van Peer, P. (Eds.), Fourth International Symposium "The Mesolithic in Europe." Leuven University Press, Leuven, pp. 153–160.

Leonard, J., Carles, V., Fox-Dobbs, K., Koch, P., Wayne, R., Van Valkenburgh, B., 2007. Megafaunal Extinctions and the Disappearance of a Specialized Wolf Ecomorph. Curr. Biol.

17, 1146–1150. doi:10.1016/j.cub.2007.05.072

Leonard, J.A., Wayne, R.K., Wheeler, J., Valadez, R., Guilén, S., Vilà, C., 2002. Ancient DNA Evidence for Old World Origin of New World Dogs. Science 298, 1613–1616.

Martins, J., Carvalho, A., Monge Soares, A., 2003. A calibração das datas de radiocarbono dos esqueletos humanos de Muge, Promontoria, Revista do Departamento de História, Arqueologia e Património da Universidade do Algarve. Departamento de história, arqueologia e património da Universidade do Algarve.

Martins, J.M.M., Carvalho, A.F., Soares, A.M.M., 2008. A calibração das datas de radiocarbono dos esqueletos humanos de Muge. Promontoria, Rev. do Dep. História, Arqueol. e Património da Univ. do Algarve Ano 6, 73–93.

Matsubayashi, J., Ohta, T., Takahashi, O., Tayasu, I., 2017. Reconstruction of the extinct Ezo wolf's diet. J. Zool. 302, 88–93. doi:10.1111/jzo.12436

Monge Soares, A.M., Gutiérrez-Zugasti, I., González-Morales, M., Matos Martins, J.M., Cuenca-Solana, D., Bailey, G.N., 2016. Marine Radiocarbon Reservoir Effect in Late Pleistocene and Early Holocene Coastal Waters off Northern Iberia. Radiocarbon 58, 869–883. doi:10.1017/RDC.2016.71

Moreno-Garcia, M., Pimenta, C., Martinez-Sanchez, R., Barroso, I., Pimenta, V., Santos, N., 2016. Caracterização osteométrica das populações atuais de lobo-ibérico Canis lupus signatus - um alicerce para o seu reconhecimento no registo arqueozoológico (Poster), in: IV Congresso Ibérico Do Lobo. Castelo Branco.

Morey, D.F., Jeger, R., 2015. Paleolithic dogs: Why sustained domestication then? J. Archaeol. Sci. Reports 3, 420–428. doi:10.1016/j.jasrep.2015.06.031

Newsome, T.M., Boitani, L., Chapron, G., Ciucci, P., Dickman, C.R., Dellinger, J.A., López-Bao, J. V., Peterson, R.O., Shores, C.R., Wirsing, A.J., Ripple, W.J., 2016. Food habits of the world's grey wolves. Mamm. Rev. 46, 255–269. doi:10.1111/mam.12067

Niemi, M., Sajantila, A., Ahola, V., Vilkki, J., 2018. Sheep and cattle population dynamics based on ancient and modern DNA reflects key events in the human history of the North-East Baltic Sea Region. J. Archaeol. Sci. Reports 18, 169–173. doi:10.1016/j.jasrep.2018.01.003

Ollivier, M., Tresset, A., Frantz, L., Bréhard, S., Bălășescu, A., Mashkour, M., Boroneanț, A., Pionnier-Capitan, M., Lebrasseur, O., Arbogast, R.-M., Bartosiewicz, L., Debue, K., Rabinovich, R., Sablin, M., Larson, G., Hänni, C., Hitte, C., Vigne, J.-D., 2018. Dogs accompanied humans during the Neolithic expansion into Europe (in press). Biol. Lett.

Pang, J.-F., Kluetsch, C., Zou, X.-J., Zhang, A., Luo, L.-Y., Angleby, H., Ardalan, A., Ekström, C., Sköllermo, A., Lundeberg, J., Matsumura, S., Leitner, T., Zhang, Y.-P., Savolainen, P., 2009. mtDNA data indicate a single origin for dogs south of Yangtze River, less than 16,300 years ago, from numerous wolves. Mol. Biol. Evol. 26, 2849–64. doi:10.1093/molbev/msp195

Paquet, P., Carbyn, L., 2003. Gray wolf Canis lupus and allies, in: GA, F., BC, T., JA, C. (Eds.), Wild Mammals of North America: Biology, Management, and Conservation. JHU Press,

Baltimore, Maryland, USA, pp. 483–510.

Peakall, R., Smouse, P.E., 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6, 288–295. doi:10.1111/j.1471-8286.2005.01155.x

Pilot, M., Branicki, W., Jedrzejewski, W., Goszczyński, J., Jedrzejewska, B., Dykyy, I., Shkvyrya, M., Tsingarska, E., 2010. Phylogeographic history of grey wolves in Europe. BMC Evol. Biol. 10, 104. doi:10.1186/1471-2148-10-104

Pilot, M., Greco, C., vonHoldt, B.M., Jędrzejewska, B., Randi, E., Jędrzejewski, W., Sidorovich, V.E., Ostrander, E.A., Wayne, R.K., 2014. Genome-wide signatures of population bottlenecks and diversifying selection in European wolves. Heredity (Edinb). 112, 428–42. doi:10.1038/hdy.2013.122

Pilot, M., JEDRZEJEWSKI, W., BRANICKI, W., SIDOROVICH, V.E., JEDRZEJEWSKA, B., STACHURA, K., FUNK, S.M., 2006. Ecological factors influence population genetic structure of European grey wolves. Mol. Ecol. 15, 4533–4553. doi:10.1111/j.1365-294X.2006.03110.x

Pilot, M., Malewski, T., Moura, A.E., Grzybowski, T., Oleński, K., Ruść, A., Kamiński, S., Ruiz Fadel, F., Mills, D.S., Alagaili, A.N., Mohammed, O.B., Kłys, G., Okhlopkov, I.M., Suchecka, E., Bogdanowicz, W., 2015. On the origin of mongrels: evolutionary history of free-breeding dogs in Eurasia. Proceedings. Biol. Sci. 282, 20152189. doi:10.1098/rspb.2015.2189

Pires, A.E., Amorim, I., Borges, C., Simões, F., Teixeira, T., Quaresma, A., Petrucci-Fonseca, F., Matos, J., 2017a. New insights into the genetic composition and phylogenetic relationship of wolves and dogs in the Iberian Peninsula. Ecol. Evol. 7, 4404–4418. doi:doi.org/10.1002/ece3.2949

Pires, A.E., Detry, C., Fernandez-Rodriguez, C., Valenzuela-Lamas, S., Arruda, A.M., De Grossi Mazzorin, J., Ollivier, M., Hänni, C., Simões, F., Ginja, C., 2017b. Roman dogs from the Iberian Peninsula and the Maghreb - A glimpse into their morphology and genetics. Quat. Int. 471 part A, 132–146. doi:10.1016/j.quaint.2017.11.044

Pires, A.E., Ouragh, L., Kalboussi, M., Matos, J., Petrucci-Fonseca, F., Bruford, M.W., 2006. Mitochondrial DNA sequence variation in Portuguese native dog breeds: diversity and phylogenetic affinities. J. Hered. 97, 318–30. doi:10.1093/jhered/esl006

Rambaut A., Suchard M.A., Xie D., Drummond A.J., 2014. Tracer | BEAST [WWW Document]. URL http://beast.bio.ed.ac.uk/tracer (accessed 7.14.17).

Ramsey, C.B., 2009. Bayesian Analysis of Radiocarbon Dates. Radiocarbon 51, 337–360.

Reimer, P.J., Bard, E., Bayliss, A., Beck, J.W., Blackwell, P.G., Ramsey, C.B., Buck, C.E., Cheng, H., Edwards, R.L., Friedrich, M., Grootes, P.M., Guilderson, T.P., Haflidason, H., Hajdas, I., Hatté, C., Heaton, T.J., Hoffmann, D.L., Hogg, A.G., Hughen, K.A., Kaiser, K.F., Kromer, B., Manning, S.W., Niu, M., Reimer, R.W., Richards, D.A., Scott, E.M., Southon, J.R., Staff, R.A., Turney, C.S.M., van der Plicht, J., 2013. IntCal13 and Marine13 Radiocarbon Age Calibration Curves 0–50,000 Years cal BP. Radiocarbon 55, 1869–1887.

doi:10.2458/azu_js_rc.55.16947

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–4.

Salewski, V., Watt, C., 2017. Bergmann's rule: a biophysiological rule examined in birds. Oikos 126. doi:10.1111/oik.03698

Sardella, R., Bertè, D., Iurino, D.A., Cherin, M., Tagliacozzo, A., 2014. The wolf from Grotta Romanelli (Apulia, Italy) and its implications in the evolutionary history of Canis lupus in the Late Pleistocene of Southern Italy. Quat. Int. 328, 179–195. doi:10.1016/j.quaint.2013.11.016

Shannon, L.M., Boyko, R.H., Castelhano, M., Corey, E., Hayward, J.J., Mclean, C., White, M.E., Said, M.A., Anita, B.A., Bondjengo, N.I., Calero, J., Galov, A., Hedimbi, M., Imam, B., Khalap, R., Lally, D., Masta, A., Oliveira, K.C., Pérez, L., Randall, J., Tam, N.M., Trujillo-Cornejo, F.J., Valeriano, C., Sutter, N.B., Todhunter, R.J., Bustamante, C.D., Boyko, A.R., 2015. Genetic structure in village dogs reveals a Central Asian domestication origin. Proc. Natl. Acad. Sci. 112, 13639–13644. doi:10.1073/pnas.1516215112

Stjerna, R.P., 2016. On death in the Mesolithic : or the mortuary practices of the last hunter-gatherers of the south-western Iberian Peninsula, 7th-6th Millennium BCE. Department of Archaeology and Ancient History, Uppsala University.

Stronen, A. V, Navid, E.L., Quinn, M.S., Paquet, P.C., Bryan, H.M., Darimont, C.T., 2014. Population genetic structure of gray wolves (Canis lupus) in a marine archipelago suggests island-mainland differentiation consistent with dietary niche. BMC Ecol. 14, 11. doi:10.1186/1472-6785-14-11

Svensson, E.M., Anderung, C., Baubliene, J., Persson, P., Malmström, H., Smith, C., Vretemark, M., Daugnora, L., Götherström, A., 2007. Tracing genetic change over time using nuclear SNPs in ancient and modern cattle. Anim. Genet. 38, 378–383. doi:10.1111/j.1365-2052.2007.01620.x

Tchernov, E., Valla, F.F., 1997. Two New Dogs, and Other Natufian Dogs, from the Southern Levant. J. Archaeol. Sci. 24, 65–95.

Thalmann, O., Shapiro, B., Cui, P., Schuenemann, V.J., Sawyer, S.K., Greenfield, D.L., Germonpré, M.B., Sablin, M. V, López-Giráldez, F., Domingo-Roura, X., Napierala, H., Uerpmann, H.-P., Loponte, D.M., Acosta, a a, Giemsch, L., Schmitz, R.W., Worthington, B., Buikstra, J.E., Druzhkova, a, Graphodatsky, a S., Ovodov, N.D., Wahlberg, N., Freedman, a H., Schweizer, R.M., Koepfli, K.-P., Leonard, J. a, Meyer, M., Krause, J., Pääbo, S., Green, R.E., Wayne, R.K., 2013. Complete mitochondrial genomes of ancient canids suggest a European origin of domestic dogs. Science 342, 871–4. doi:10.1126/science.1243650

Umbelino, C.I.S., 2006. Outros sabores do passado: as análises de oligoelementos e de isótopos estáveis na reconstituição da dieta das comunidades humanas do Mesolítico Final e do Neolítico Final-Calcolítico do território português (PhD thesis). Universidade de Coimbra.

Verginelli, F., Capelli, C., Coia, V., Musiani, M., Falchetti, M., Ottini, L., Palmirotta, R.,

Tagliacozzo, A., De Grossi Mazzorin, I., Mariani-Costantini, R., 2005. Mitochondrial DNA from prehistoric canids highlights relationships between dogs and South-East European wolves. Mol. Biol. Evol. 22, 2541–51. doi:10.1093/molbev/msi248

Vigne, J.-D., 2005. L'humérus de chien magdalénien de Erralla (Gipuzkoa, Espagne) et la domestication tardiglaciaire du loup en Europe. MUNIBE 57, 279–287.

Vis, G.-J., Kasse, C., Vandenberghe, J., 2008. Late Pleistocene and Holocene palaeogeography of the Lower Tagus Valley (Portugal): effects of relative sea level, valley morphology and sediment supply. Quat. Sci. Rev. 27, 1682–1709. doi:10.1016/J.QUASCIREV.2008.07.003

von den Driesch, A., 1976. A Guide to the Measurement of Animal Bones from Archaeological Sites. Peabody Museum of Archaeology and Ethnology, Harvard University.

Wang, G.-D., Zhai, W., Yang, H.-C., Wang, L., Zhong, L., Liu, Y.-H., Fan, R.-X., Yin, T.-T., Zhu, C.-L., Poyarkov, A.D., Irwin, D.M., Hytönen, M.K., Lohi, H., Wu, C.-I., Savolainen, P., Zhang, Y.-P., 2016. Out of southern East Asia: the natural history of domestic dogs across the world. Cell Res. 26, 21–33. doi:10.1038/cr.2015.147

Zilhão, J., 2001. Radiocarbon evidence for maritime pioneer colonization at the origins of farming in west Mediterranean Europe. Proc. Natl. Acad. Sci. 98, 14180–14185.

Data Accessibility:

Partial mtDNA sequences of ancient *Canis* remains were deposited in GenBank, namely for the six Mesolithic dogs (accession numbers KY014675-77, KY014682-83 and KY014652), two Palaeolithic wolves (KY014650-51), and one Chalcolithic wolf (KY014649); as well as for the additional 37 dog remains from other periods, namely Neolithic (KY014653, KY014667), Chalcolithic (KY014654-66, KY014668-71 and KY014680), Roman (KY014672-74 and KY014684-94, (Pires et al., 2017b)) and Late Antiquity (KY014678-79 and KY014681). An alignment of these sequences together with haplotypes from extant dogs and wolves from Iberia is available from

JAS_SequenceAlignment_TheCuriousCaseoftheMesolithicIberianDogs_AEPiresetal (fasta file).

Author Contributions

A.C. Sousa, M. Moreno-Garcia, A. M. Arruda, E. Porfírio, A. Valente, C. Fernandez-Rodriguez, N. Bicho, J.M. Arnaud, P. Arias, M. Diniz, J.L. Cardoso carried out field work, chronology determination and/or provided biometric data; C. Detry and S. Davis did the zooarchaeological analysis; A. C. Araújo undertook ^{14}C calibrations and supplied Fig. 2A and B; F. Petrucci-Fonseca provided road-killed or recovered illegally hunted wolf carcasses for the extant wolf skeletal collections; S. Davis, M. Moreno-Garcia and others prepared the wolf and dog reference collections in the LARC and MUHNAC; A.L. Alves prepared ULHT dog skeleton reference collection; A.E. Pires, F. Simões, I.R. Amorim and J. Matos provided genetic data from extant dogs and wolves, A.E. Pires, C. Ginja and M. Ollivier were responsible for the ancient DNA laboratory work, data validation and bioinformatics analysis; C. Hänni and A. Götherström provided the laboratory facilities to carry out ancient DNA analysis and acted as consultants; D. Gonçalves performed the statistical analysis for biometric data; C. Ginja, L. Chikhi and R. Rasteiro undertook population genetics analyses, interpretation of the results and contributed to the writing of the paper; A.E. Pires, C. Detry, C. Ginja and L. Chikhi wrote the paper and all authors read and contributed comments to the work.