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(Article begins on next page)

# **BIRDS OF PREY AND HUMANS IN PREHISTORIC EUROPE: A VIEW FROM EL MIRÓN CAVE, CANTABRIA (SPAIN).**

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## **ABSTRACT**

Bird eggs can become part of the archaeological record either accidentally or as a result of human activities but, in both instances, they can reveal important aspects of the environment at the site, the ways in which people chose to exploit it, and even the existence of subtle ecological balances between humans and other animals. This is the case for El Mirón, one of the most important cave sites in Cantabrian Spain, with occupation levels spanning around 40,000 years, from the late Middle Palaeolithic to the Bronze Age. This mountainous area in Cantabria was an ideal environment for hunting medium-sized game and, as such, supported both human and non-human predators, including birds of prey.

Here we use a combination of peptide mass fingerprinting (by MALDI-MS) and protein sequencing (by LC-MS/MS) in order to taxonomically identify ninety-five fragments of eggshells recovered from nineteen archaeological layers. We firmly identify these as diurnal birds of prey (Accipitridae) and suggest that the species might have been Bearded vulture, based on previous taphonomic studies that highlighted its presence at the cave. The implication is that both species of diurnal predators, humans and birds, inhabited the cave and used the surrounding environment during different periods of the year.

**KEYWORDS:** Avifauna; Birds of prey; Zooarchaeology; Mass Spectrometry (ZooMS); Paleolithic; El Mirón Cave, Cantabrian Spain

## **INTRODUCTION**

Birds are liminal animals, connecting sky and earth, and as such have inhabited both the real and the symbolic worlds of humans since early prehistory (Serjeantson, 2009). It is not a coincidence that one of the few pictorial representations of the human figure in parietal prehistoric art, in the “Shaft of the dead man” at Lascaux, is a bird-man fighting a bison, and the whole scene is enigmatically observed by a bird on a pole (Lewis-Williams, 2002). Among all avifauna, birds of prey occupy a special place in the mental landscape during prehistory, as testified by the increasing number of findings of raptors’ and corvids’ claws and bones bearing cut-marks typical of feather-removing, or used as musical instruments, both in Middle and Upper Palaeolithic sites (Conard et al., 2009; Finlayson et al., 2012; Hardy and Moncel, 2011; Kuhn et al., 2001; Morin and Laroulandie, 2012; Peresani et al., 2011; Romandini et al.,

2014). However, the preservation bias affecting bird remains, as well as the lack of widespread iconographic representations, have consistently hindered all efforts to gain a deeper understanding of the reciprocal relationship between humans and birds in the past.

In this study we apply a novel methodology for taxonomic identification of bird eggshell remains based on the analysis of proteins preserved within the eggshell calcite crystals (Presslee et al., 2018). In order to assess whether eggshell can add another dimension of understanding to archaeo-faunal assemblages and, by extension, to how humans behaved in a certain landscape in the past, we focus on a long archaeological sequence from Cantabria, Northern Spain, spanning ~40,000 years from the late Middle Palaeolithic to the Bronze Age in the cave of El Mirón.

El Mirón is a large, west-facing cave located at 260 m a.s.l. on a cliff in a foothill range (1000 m a.s.l.) of the Cantabrian Cordillera near the edge of the Basque Region c. 20 km from the Holocene shore of the Bay of Biscay (c. 25 km from the Last Glacial shore) (Figure 1). It consists of a spacious, dry, fully sunlit vestibule (30 m-deep x 8-16 m-wide and 13-20 m-high) and a narrower, higher, 100 m-long, dark inner cave, filled to the roof with alluvial sediments at its rear. The principal excavations (directed by Gonzalez Morales and Straus between 1996-2013) consisted of a 9.25 m<sup>2</sup> block in the outer vestibule, a 10.5 m<sup>2</sup> block in the vestibule rear with two contiguous extensions: a 2 m<sup>2</sup> sondage, dug from the base of a large clandestine pothole, and a 4 m<sup>2</sup> area in the space between a large roof-fall block and the south-east corner of the vestibule, where a 19,000 year-old human burial was discovered (Straus & González Morales, 2012; Straus et al., 2015a and 2015b). The outer and rear vestibule excavation areas were connected by a 9-m-long, 0.5-1.0-m-wide connecting trench. The archaeological sequence in the outer vestibule consists of levels pertaining to the Lower, Middle and Upper Magdalenian, Azilian, Mesolithic, early and late Neolithic, Chalcolithic and early Bronze Age. All but the Upper Magdalenian, Azilian and Mesolithic levels are extremely rich in archaeological materials. The Lower Magdalenian through Neolithic layers are represented in the mid-vestibule trench, and the whole Magdalenian sequence, plus Solutrean, Gravettian and Mousterian levels are present in the vestibule rear. There are traces of Medieval uses of the cave (dated torch fragments) both in the vestibule and in a test trench in the inner cave, which also has evidence of Lower Magdalenian occupation. The archaeological levels are dated by 92 radiocarbon assays ranging from >45,000-500 years ago.

The human burial is of a robust, apparently healthy, 35-40-year-old female, who had a mixed diet including terrestrial meat (the majority), seafood and plant foods (including mushrooms). She is the only interment known from the Magdalenian of the Iberia and only the second one from the entire Upper Palaeolithic of the Peninsula. Her body and/or later her bones had been stained with non-local hematite ochre. She was buried in tight foetal position in a small hole dug into a rich Lower Magdalenian layer between a 2x1x1 m roof-fall block and the cave wall. Her cranium and most of her long bones had been removed in an apparent reburial after disturbance by a carnivore (Marín-Arroyo, 2015). Her grave seems to have been “marked” by engravings (including a multi-line inverted [pubic?] triangle) on the outer (western) face of the block and the inner face, contiguous with the burial, is stained red with the same ochre (Straus et al., 2015).

El Mirón represents an ideal case study because the important, yet elusive, presence of birds has been posited in the past. The analysis of bird bones has been published only for the

Holocene layers, from the Mesolithic to the Bronze Age (Elorza, 2012), and showed the presence of the following species: *Accipiter gentilis* (northern goshawk), *Falco* sp. (falcons), cf. *Tetrao tetrix* (black grouse), *Columba livia/oenas* (rock dove/stock dove), *Columba palumbus* (wood pigeon), *Strix aluco*, (tawny owl) *Asio* sp. (true owl), *Turdus* sp. (thrush), *Coccothraustes coccothraustes* (hawfinch), *Pyrrhocorax graculus* (alpine chough), *Corvus corax* (common raven). Bird bones are relatively abundant in the Mousterian and Early Upper Palaeolithic levels (Marín-Arroyo et al., 2018), present in modest numbers in the Solutrean levels, and very rare in the Magdalenian and Azilian ones (M. Carvalho; J.M. Geiling; E.L. Jones, pers. comm., Oct 2018). In general, the frequency of bird bones seems to be inversely related to the abundance and density of cultural and macromammal remains in the cave; the scanty, ephemeral Early Upper Palaeolithic human occupations have more bird bones than the overlying layers, and some of these display carnivore gnaw marks, suggesting non-human accumulation (Marín-Arroyo et al., 2018). On the contrary, zooarchaeological analysis of the few avian remains from other caves in the Cantabrian region shows the presence of a wide variety of birds. For example, at La Riera, birds were exploited as a food resource, as testified by a species assemblage relatively rich in waterfowl and one bone with cutmarks (Eastham, 1986). Similarly, the spectacular assemblage recovered from Santa Catalina (Bay of Biscay) shows that, in the Upper Magdalenian, seabirds were heavily exploited as one of the many coastal resources (Laroulandie et al., 2016), while this was not the case for other sites, for example the Magdalenian and Solutrean levels at Las Caldas (Eastham, 2017).

The presence of birds of prey (mainly nocturnal) at El Mirón is demonstrated by taphonomic studies of the small mammal bone assemblages in the cave (Cuenca-Bescós et al., 2012). Additionally, the *diurnal* bird of prey *Gypaetus barbatus* (Bearded vulture) has been identified as an important bone accumulator agent on the basis of the clear digestion marks on specific anatomical elements of medium-sized mammals (Marín-Arroyo, 2009; Marín-Arroyo et al., 2009), such as in Corsica (Robert and Vigne, 2002) but also at Caldeirão cave (Central Portugal; Davis et al., 2007). In Hornos de la Peña (Cantabria) Harlé (1912) indicated the presence of black vulture. However, so far the direct evidence for vultures at El Mirón is limited to one black vulture humerus in the Lower Magdalenian level 17.

The study of the ninety-five eggshell fragments recovered from the sieved sediments from nineteen layers within El Mirón aims primarily to clarify the composition of the avifaunal assemblage at the site and whether this changed over time. Taxonomic identification was carried out on the basis of the analysis of the ancient proteins preserved in eggshell, using both peptide mass fingerprinting (by MALDI-MS) and proteomics (by LC-MS/MS). Our second aim was to assess whether the eggshell assemblages were due to natural (i.e. eggshell fragments falling from nests on the ceiling) or anthropic (i.e. exploitation of bird eggs by humans) deposition, by integrating the results obtained on the eggshell proteins with available zooarchaeological information and by interpreting this within the archaeological context of the cave.

### **Approximate location of figure 1**

## **MATERIALS AND METHODS**

Eggshell was recovered post-excavation by floating sediments with a 1 mm mesh and sieving with 2 mm mesh. Most of the eggshell comes from the > 2 mm fraction, and is kept in storage

at the Museum of Prehistory and Archaeology of Cantabria (MUPAC), Santander, Spain. There are only a few excavation squares where the concentration of eggshell is clear (Figure 1): square O6 from the mid-vestibule (Mesolithic, Final Magdalenian-Early Azilian, Upper Magdalenian, Lower Magdalenian); squares J4 and I4 from the Cabin area (Final Magdalenian-Early Azilian, Lower Magdalenian), T9 (Upper Magdalenian) and X10 (Solutrean, Gravettian and Early Upper Palaeolithic) from the Corral area. Only one sampled layer (503.1, square X6, above the burial of the Lower Magdalenian “Red Lady” (Straus et al., 2015)) represented an intact hearth, with ash and charcoal. The upper part of the southern wall and the east section of the vestibule, along the ramp that ascends to the cave interior, have a series of horizontal ledges suitable for nesting and almost inaccessible to humans. In the case of squares T9 and X10, at the foot of the ramp, a provenance due to erosional processes cannot be excluded.

Samples were taken for each archaeological layer available, randomly selecting five fragments per layer, for a total of ninety-five eggshell samples. Each fragment was subsampled, 15–20 mg taken, powdered, bleached for 100 hours, demineralised in cold weak 0.6 M hydrochloric acid and the extracted proteins digested with trypsin as described previously (Demarchi et al., 2016; Presslee et al., 2018). Tryptic digests were spotted in triplicate (1  $\mu$ L each) with  $\alpha$ -Cyano-4-hydroxycinnamic acid ( $\alpha$ -CHCA) matrix on a clean MTP384 Bruker ground steel MALDI target plate and analysed using a Bruker Ultraflex III MALDI-ToF/ToF mass spectrometer, with the following parameter settings: ion source, 25 kV; ion source, 21.4 kV; lens voltage, 9 kV, laser intensity 40–55% and mass range 800–4000 Da. Peptide masses below 650 Da were suppressed. Each sample was externally calibrated against an adjacent spot containing a mixture of six peptides (des-Arg1 Bradykinn  $m/z$ = 904.681, Angiotensin I  $m/z$ = 1295.685, Glu1-Fibrinopeptide B  $m/z$ = 1750.677, ACTH (1–17 clip)  $m/z$ = 2093.086, ACTH (18–39 clip)  $m/z$ = 2465.198 and ACTH (7–38 clip)  $m/z$ = 3657.929). Calibrated spectra were analysed using the open-source software mMass (Strohalm et al., 2010), averaging the three replicates for each sample after a visual check of the quality of each spectrum.

Three archaeological samples (LOTs 14139\_2, 14145\_3 and 14147\_2; LOTs indicated by an asterisk in Table 1), were also analysed by LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry): peptides were separated on a PepMAP C18 column (75  $\mu$ m  $\times$  500 mm, 2  $\mu$ m particle size, Thermo) using a Dionex Ultimate 3000 UPLC at 250 nL/min and acetonitrile gradient from 2% to 35% in 5% dimethyl sulfoxide/0.1% formic acid. Peptides were detected with a Q-Exactive mass spectrometer (Thermo) at a resolution of 70,000 @ 200  $m/z$ . Up to 15 precursors were selected for High-energy Collision Dissociation (HCD) fragmentation. Tandem mass spectra were processed using the software PEAKS v. 8.5 (Ma et al., 2003) and searched against a database containing 2,453,941 proteins, i.e. all sequences available on NCBI restricting the taxonomy to Aves (fasta database downloaded on 11/02/2018) and all common contaminants (cRAP; common Repository of Adventitious Proteins: <http://www.thegpm.org/crap/>). We note that among all sequences, the database contains 56 sequences of ovocleidin-116, a major eggshell protein, as well as the following C-lectins from a variety of species:

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>sp|P83515.1|SCAL2_STRCA RecName: Full=Struthiocalcin-2; Short=SCA-2
>sp|P83514.1|SCAL1_STRCA RecName: Full=Struthiocalcin-1; Short=SCA-1
>sp|Q9PRS8.2|OC17_CHICK RecName: Full=Ovocleidin-17; Short=OC-17
>sp|P84616.2|DCAL2_DRONO RecName: Full=Dromaiocalcin-2; Short=DCA-2
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>sp|P84615.2|DCAL1\_DRONO RecName: Full=Dromaiocalcin-1; Short=DCA-1  
 >sp|P84618.2|RCAL2\_RHEAM RecName: Full=Rheacalcin-2; Short=RCA-2  
 >sp|P84617.2|RCAL1\_RHEAM RecName: Full=Rheacalcin-1; Short=RCA-1  
 >XP\_011597343.1 PREDICTED: ansocalcin-like [*Aquila chrysaetos canadensis*]  
 >XP\_013801307.1 PREDICTED: rheacalcin-1-like, partial [*Apteryx australis mantelli*]  
 >XP\_005443542.1 PREDICTED: rheacalcin-2-like [*Falco cherrug*]  
 >XP\_005243559.1 PREDICTED: rheacalcin-2-like [*Falco peregrinus*]  
 >XP\_011597342.1 PREDICTED: rheacalcin-2-like [*Aquila chrysaetos canadensis*]  
 >XP\_010564359.1 PREDICTED: rheacalcin-2-like [*Haliaeetus leucocephalus*]  
 >XP\_021238989.1 dromaiocalcin-1-like [*Numida meleagris*]  
 >XP\_021238988.1 dromaiocalcin-1-like [*Numida meleagris*]  
 >XP\_014746250.1 PREDICTED: dromaiocalcin-1-like [*Sturnus vulgaris*]  
 >XP\_014118214.1 PREDICTED: dromaiocalcin-1-like [*Pseudopodoces humilis*]  
 >XP\_013801306.1 PREDICTED: struthiocalcin-2 [*Apteryx australis mantelli*]  
 >XP\_012984941.1 PREDICTED: struthiocalcin-2-like, partial [*Melopsittacus undulatus*]  
 >XP\_012984941.1 PREDICTED: struthiocalcin-2-like, partial [*Melopsittacus undulatus*]  
 >pdb|1GZ2|A Chain A, Crystal Structure Of The Ovocleidin-17 A Major Protein Of The Gallus Gallus Eggshell Calcified Layer.  
 >AHA91755.1 ovocleidin-17 [*Gallus gallus*]  
 >AAB35101.1 ovocleidin 17, OC-17=17 kda matrix protein {N-terminal} [chickens, White Leghorn, eggshells, Peptide Partial, 24 aa]  
 >pir|S78596 ovocleidin - chicken  
 >XP\_021238987.1 ovocleidin-17-like [*Numida meleagris*]

The search was performed without indicating trypsin as the digestion enzyme, i.e. performing a “no enzyme” search, in order to identify cleavages due to natural hydrolysis as well as those due to the proteolytic enzyme used (trypsin). Parent ion and fragment ion mass tolerance were set to 5 ppm and 0.05 Da, respectively. The following threshold values were used for acceptance of high-quality peptides: false discovery rate threshold 0.5%, protein scores  $-10\lg P \geq 20$ , number of unique peptides  $\geq 2$ , de novo sequences scores (ALC %)  $\geq 80$ . The mass spectrometry proteomics datasets have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010439.

Lot Number	Excavation Area	Level and <sup>14</sup> C date (Straus and Morales, 2003)	Square	Chronology	Number of fragments
14134	Mid-Vestibule	304	O6	Mesolithic	5
14135	Mid-Vestibule	305 (10,362-9818 cal BC)	Q7	Azilian	5
14136	Cabin	11 (level 11.1: 12,039-11,523 cal BC)	J4	Final Magdalenian-Early Azilian	5
14137	Mid-Vestibule	306 (11,861-11,525 cal BC)	O6	Final Magdalenian-Early Azilian	5
14138	Mid-Vestibule	306 (11,861-11,525 cal BC)	O6	Final Magdalenian-Early Azilian	5
14139 *	Mid-Vestibule	307	O6	Upper Magdalenian	5
14140	Mid-Vestibule	308 (13,403-12,166 cal BC)	O6	Upper Magdalenian	5
14141	Corral	106.1	T9	Upper Magdalenian	5
14142	Corral	107.2	T9	Upper Magdalenian	5
14143	Mid-Vestibule	309	O6	Magdalenian	5
14144	Mid-Vestibule	311.1	O6	Lower Magdalenian	5
14145 *	Corral	503.1	X6	Lower Magdalenian	5
14146	Cabin	14	I4	Lower Magdalenian	5
14147 *	Cabin	15 (16,679-15,810 cal BC)	J4	Lower Magdalenian	5

14148	Cabin	16 (16,487-15,910 cal BC)	I4	Lower Magdalenian	5
14149	Cabin	17 (16,852-16,184 cal BC)	J4	Lower Magdalenian	5
14150	Corral	126 (21,070-20,010 cal BC)	X10	Solutrean	5
14151	Corral	128 (29,940-29,460 cal BC)	X10	Gravettian	5
14152	Corral	129	X10	Early Upper Palaeolithic	5

**Table 1: Details of the 95 eggshell samples analysed in this study. Note that the layers investigated span the period between the Early Upper Palaeolithic and the Mesolithic.**

## RESULTS AND DISCUSSION

### MALDI-MS AND LC-MS/MS DATASETS

The MALDI spectra (all available in Appendix 1) showed that at least two species of birds were present at the site in different periods. The first species is represented by only three of the samples (LOTs 14152-2, 14152-4, 14152-5), all from the Early Upper Palaeolithic level. The spectra were similar to each other (Figure 2A), with intense peaks at  $m/z$  1666.8 and 1723.8, which could be matched to peptide sequences of ansocalcin from *Anas* sp. using the reference dataset of Presslee et al. (2018). It is likely that these three eggshells belong to a species of Anatidae, although further analyses will be carried out in future to refine this identification.

The second species is represented by the spectra obtained on the remaining ninety-two eggshell fragments. These were very similar to each other, with the main values typically falling at  $m/z$  832.5, 1037.5, 1158.6, 1803.9, 1847.8, 1931.9, 2087.9 (example shown in Figure 2B). Some samples (LOTs 14134-2, 14136-1, 14137-2, 14137-3, 14137-5, 14150-1, 14150-2, 14150-3, 141451-1, 14151-2, 14151-3, 14151-4, 14152-1, 14152-3) yielded low-intensity signals (example shown in Figure 2C) but the major peaks still included  $m/z$  832.5, 1847.8, 1931.9. The MALDI spectra for these ninety-two samples could not be interpreted on the basis of the peptide markers identified in the study of Presslee et al. (2018), indicating that the taxa represented at the cave likely belong to avian families not included in the Presslee et al.'s reference collection. In order to identify this taxon, which clearly dominates the assemblage, we selected three representative biological replicates (LOTs 14139\_2, 14145\_3, 14147\_2) for sequencing by LC-MS/MS.

### **Approximate location of figure 2**

The three eggshell fragments analysed by LC-MS/MS yielded between 1500 and 2500 peptide-spectrum matches (Table 2; search results are reported in Appendix 2).

In sample 14139\_2, the top ten proteins (coverage > 40%) all produced hits against the proteomes of *Aquila chrysaetos canadensis* (American golden eagle; 6 hits), *Haliaeetus leucocephalus* (Bald eagle; 3 hits) and *Balearica regulorum gibbericeps* (Grey-crested crane; 1 hit). The major protein types identified were all common *intracrystalline* eggshell proteins: rheocalcin-like and ansocalcin-like. These are C-type lectins, involved in mineralization: the first exhibits 61% sequence identity with struthiocalcin-2 and the second 58% identity with struthiocalcin-1 (Mann and Siedler, 2004). Other major proteins are: BPI fold-containing family B member 4-like, bactericidal permeability-increasing proteins that are linked to lipid binding;

ovomucoid-like, which is an egg-white protein; matrix extracellular phosphoglycoprotein (MEPE), which is the orthologue of ovocleidin-116 (Bardet et al., 2010); cystatin-like protein, i.e. a proteinase inhibitor protein of the cystatin superfamily (Gerhartz et al., 1997; Rose-Martel et al., 2012). Similarly, the top proteins of sample 14145\_3 (ovomucoid-like, ansocalcin-like, rheacalcin-like, BPI-fold containing family member 4-like) matched the proteomes of the American golden eagle (4 hits), the Bald eagle (2 hits), the Red-throated dive (*Gavia stellata*, 1 hit), the Grey-crested crane (1 hit) and the Dalmatian pelican (*Pelecanus crispus*; 1 hit). With regard to sample 14147\_2, the top-identified proteins were rheacalcin-like, MEPE-like, ansocalcin-like, BPI-fold containing family member 4-like and collagen alpha1(XII) chain, all from *H. leucocephalus*. However, some matches were also found with collagen, mucins, BPI-fold containing proteins, tubulin and other highly conserved sequences from *Phaeton lepturus* (White-tailed tropicbird), *Corvus brachyrhynchos* (American crow), *Acanthisitta chloris* (Rifleman).

Statistics	LOT 14139_2	LOT 14145_3	LOT 14147_2
Peptide-Spectrum Matches	2476	1465	2298
Peptide sequences	1517	536	1520
Proteins	177 (#Unique Peptides: 63 (>2); 114 (=2))	117 (#Unique Peptides: 26 (>2); 91 (=2))	363 (#Unique Peptides: 199 (>2); 164 (=2))
De Novo Only Spectra	1151	686	718
Top proteins (% coverage)	Rheacalcin-2-like [ <i>Aquila chrysaetos canadensis</i> ] (58%); Rheacalcin-2-like [ <i>Haliaeetus leucocephalus</i> ] (58%)	Ovomucoid-like [ <i>Aquila chrysaetos canadensis</i> ] (70%)	Rheacalcin-2-like [ <i>Aquila chrysaetos canadensis</i> ] (62%)
Top organism (top proteins frequency)	<i>Aquila chrysaetos canadensis</i>	<i>Aquila chrysaetos Canadensis</i>	<i>Haliaeetus leucocephalus</i>

**Table 2: Summary of protein identification results obtained by LC-MS/MS analysis of three eggshell samples from El Mirón.**

### TAXONOMIC IDENTIFICATION

The bioinformatic analysis of the LC-MS/MS data was carried out using a database comprising all available bird sequences available at the time (February 2018), including the 48 annotated bird genomes spanning 32 orders (including all 30 neognaths) released in 2014 (Jarvis et al., 2014; Zhang et al., 2014). All main hits obtained related to either *Aquila* or *Haliaeetus*, belonging to family Accipitridae, and not to any of the close relatives included in the database, e.g. Falconidae or Cathartidae. Therefore, we can exclude the possibility that the eggshells belonged to non-Accipitriformes. However, we cannot refine our identification within the family Accipitridae as the genomes available for this family are limited to *Aquila* and *Haliaeetus*; thus, the proteomic approach can currently only provide classification to the level of family. Such



refinement will be possible in the future: at the time of writing, genomes are being constantly added to the public databases, with a plan of sequencing approximately 300 bird families and subfamilies and, ultimately, all avian species (<https://b10k.genomics.cn>).

The peptide sequences obtained by database searches of the tandem mass spectra were used to obtain potential Accipitridae “marker”  $m/z$  values. Firstly, the MS data (forty-five spectra) for LOTs 14139, 14145 and 14147 were averaged and the  $m/z$  values with signal-to-noise ratios  $> 6$  obtained from this combined dataset matched with the peptide sequences identified by LC-MS/MS. This resulted in 100 *potential* “markers” for Accipitridae. Of these, 32 peptide sequences could be assigned to ansocalcin-like from *A. chrysaetos canadensis* or the homologue carnitine O-palmitoyltransferase 1, muscle isoform from *H. leucocephalus*; 19 to rheacalcin-2-like from *A. chrysaetos canadensis*. Others could be assigned to sequences of mucins, collagen, egg white and BPI-fold-containing proteins; however, these tend to be highly conserved among birds and are therefore less useful for taxonomic identification. A BLASTp search was performed for each of the peptides in order to identify their occurrence in taxa other than Accipitridae. Appendix 2 contains a list of the 100 peptides, highlighting those that can presently be considered as Accipitridae-specific, i.e. that were not found in non-Accipitridae birds.

It is important to note that while these “marker” peptide sequences can provide family-level identification, the  $m/z$  values alone may not: it is possible that the same  $m/z$  value (within error) corresponds to several (isobaric) peptide sequences. For example,  $m/z$  1257.8, one of the main peaks found in the El Mirón MALDI-MS dataset, can be assigned to sequence SAWEGDDPPKR from carnitine O-palmitoyltransferase (*Haliaeetus leucocephalus*) but can also be found (within a 0.2 Da error) in the reference marker list of Presslee et al. (2018) for *Carduelis*, *Passer* and *Muscicapa*. Table 3 therefore only reports the 29  $m/z$  values and associated peptide sequences that can currently be considered Accipitridae markers. Figure 3 shows the product ion spectra for six selected peptides, which are commonly found in the El Mirón MALDI-MS dataset.

<b><i>m/z</i></b>	<b><i>Peptide sequence (with modifications)</i></b>	<b><i>Protein ID</i></b>
832.5	ALAAFIAR	Ansocalcin-like [ACC]
923.4	SVHSVEEH	Rheacalcin-2-like [ACC]
943.4	WEGDDPPK	Ansocalcin-like [ACC]
1003.5	GC(+57.02)YGFFPR	Rheacalcin-2-like [ACC]
1037.5	RAE AFC(+57.02)QR	Rheacalcin-2-like [ACC]
1038.5	RAE AFC(+57.02)Q(+.98)R	Rheacalcin-2-like [ACC]
1101.5	SAWEGDDPPK	Ansocalcin-like [ACC]
1158.6	N(+.98)VWIGLYHR	Ansocalcin-like [ACC]
1243.6	AAGKEVC(+57.02)QRPK	Mucin-5AC-like [HL]
1263.6	LASVHSVEEHR	Rheacalcin-2-like [ACC]
1289.6	LASLHTPEEHR	Ansocalcin-like [ACC]
1365.7	E(-18.01)EHRALAAFIAR	Ansocalcin-like [ACC]
1408.7	FED(+14.02)GC(+57.02)YGFFPR	Rheacalcin-2-like [ACC]
1463.7	RHPELSTQ(+.98)LILR	Serum albumin [ACC]
1527.7	E(-18.01)EEN(+.98)VWIGLYHR	Ansocalcin-like [ACC]

1564.7	YSAW(+15.99)EGDDPPK	Carnitine O-palmitoyltransferase 1, muscle isoform [HL]
1567.7	SQAWM(+15.99)WVDGSQTR	Ansocalcin-like [ACC]
1568.7	SQ(+.98)AWM(+15.99)WVDGSQTR	Ansocalcin-like [ACC]
1571.7	C(+42.01)HLASLHTPEEHR	Ansocalcin-like [ACC]
1785.8	E(-18.01)EEEEEN(+.98)VWIGLYHR	Ansocalcin-like [ACC]
1795.8	REEEEEEN(+.98)VWIGLY	Ansocalcin-like [ACC]
1803.9	EEEEEN(+.98)VWIGLYHR	Ansocalcin-like [ACC]
1847.8	GWVPFED(+14.02)GC(+57.02)YGFFPR	Rheacalcin-2-like [ACC]
1914.9	E(-18.01)EEEEEN(+.98)VWIGLYHR	Ansocalcin-like [ACC]
1931.9	EEEEENVWIGLYHR	Ansocalcin-like [ACC]
1932.9	EEEEEN(+.98)VWIGLYHR	Ansocalcin-like [ACC]
2087.9	REEEEEENVWIGLYHR	Ansocalcin-like [ACC]
2088.9	REEEEEEN(+.98)VWIGLYHR	Ansocalcin-like [ACC]
2104.9	REEEEEEN(+.98)VW(+15.99)IGLYHR	Ansocalcin-like [ACC]

**Table 3: Accipitridae markers frequently found in El Mirón eggshells. ACC = [*Aquila chrysaetos canadensis*]; [HL] = [*Haliaeetus leucocephalus*]**

### Approximate location of Figure 3

## DISCUSSION

We found that the preservation of the intracrystalline proteins is overall very good, and this study is the first to report proteomics-based taxonomic identification of eggshell from a Paleolithic site from a relatively temperate environment (at Ramales de la Victoria temperatures typically vary between a maximum of 28°C and a minimum of 5°C, although these variations will be considerably dampened in the cave). We also note that Pleistocene temperatures were certainly colder.

The main sequences identified in the eggshell typically display very acidic (Glu-rich) domains, including an EEEEEENVWIGLYHR ( $m/z = 1931.9$ ) peptide from an ansocalcin-like protein. Peptide YSAWEGDDPPK from ansocalcin is also present among the main peptides detected by both MALDI-MS and LC-MS/MS. The domain ALDDDDYPK from the sequence of struthiocalcin-1, which corresponds to YSAWEGDDPPK in an alignment of the two proteins, is preserved in fossil ostrich eggshell for up to 3.8 Ma (Demarchi et al., 2016). Peptides ALAAFIAR ( $m/z = 832.5$ , ansocalcin-like) and GWVPFEDGCYGFPR ( $m/z = 1847.8$ , rheacalcin-2-like) are among the most stable and are typically found in the spectra of the most degraded eggshell. Poor-quality spectra were obtained mainly for some of the oldest samples (LOTs 14150-14151-14152, from the Solutrean, Gravettian and Early Upper Palaeolithic layers of the Corral Area, respectively). However, three of the samples from LOT 14137 (Mid-Vestibule, Final Magdalenian-Early Azilian) also yielded low-intensity spectra.

Before discussing the archaeological relevance of the results, it is important to state clearly that our dataset is affected by inherent biases:

1. Thicker eggshells, such as that of large birds of prey, survive better than thinner, smaller eggs laid by, for example, small Passeriformes, which are therefore under-represented in the faunal assemblage;

2. The recovery of the eggshell during and post-excavation varies according to excavation season and operator;
3. The relative paucity of avian genomic data limits our ability to identify birds beyond the family/order level in most cases, including for Accipitridae (genomes available: *Aquila chrysaetos*, *Haliaeetus leucocephalus*, *Haliaeetus albicilla*).
4. The use of two different analytical techniques, namely MALDI-MS and LC-MS/MS, may result in the over- or under-representation of certain charged species / peptides.

Despite these caveats, the datasets obtained clearly indicate that eggshell from one main bird taxon was deposited in the cave from the Gravettian until the Mesolithic. This bird taxon can be firmly identified as a member of the Accipitridae, the diurnal birds of prey. The Early Upper Palaeolithic level yielded two fragments (14152-1 and 14152-3) that could be identified as members of this same taxon, with relatively poor-quality spectra, but also three (14152-2, 14152-4 and 14152-5) that are likely to belong to a member of the Anatidae family, based on one marker value at  $m/z$  1723.8. At this stage we did not attempt to identify this Anatidae using LC-MS/MS analyses.

Our work provides direct evidence for the presence of a species of diurnal bird of prey in the area of El Mirón. However, the identification of the species is only possible by combining the biomolecular data on the eggshell with the existing taphonomical data on the macromammal remains. For the Pleistocene levels of El Mirón, taphonomic studies of medium-sized mammal bones have highlighted the possible presence of *Gypaetus barbatus* (Marín-Arroyo et al., 2009), a bird which is known to have been present in the area, for example at Santa Catalina (Esplosin, 2014), Aitzbitarte IV, Hornos de la Peña, as well as other Iberian sites from the Upper Pleistocene until the Middle Ages (see a summary in Marín-Arroyo, 2010). This is not surprising, given the nature of the environment: mountains with caves and sheer cliff faces, ideal for nesting. If we accept this identification, then the biology and behaviour of the bird can give precious clues as to the way vultures and humans coexisted in the past, alternating the use of the same cave and exploiting the same environment. The imposing, awe-inspiring and, probably, menacing presence of these bone-eating predators at the site could have not been ignored by humans, and vice versa: where one existed, the other must have behaved carefully.

The bearded vulture or *ossifrage* (“bone breaker”) has a highly specialised diet, feeding on the bones of dead animals (Margalida and Marín-Arroyo, 2013; Marín-Arroyo and Margalida, 2012). Both Paleolithic humans and bearded vultures predated on medium-sized ungulates (southern chamois *Rupicapra pyrenaica*, Spanish ibex *Capra pyrenaica*, red deer *Cervus elaphus* and roe deer *Capreolus capreolus* (Margalida and Marín-Arroyo, 2013)), and the analysis of the remains from El Mirón showed that bearded vultures had a preference for foot limb bones (metapodials and phalanges). This is because the quality of the fatty acids involved, in terms of oleic acid percentage, is higher in these anatomical parts, resulting in easier digestibility and better nutritional value. While small bones can be ingested directly, larger bones are typically broken by throwing them to the ground from a great height; the birds often use specific ossuaries for this purpose. Interestingly, this produces the same breakage pattern as that resulting from bones being broken by humans using tools.

This behaviour must certainly have been known to the human frequenters of the cave, although it is important to try to understand the extent and level of interaction between humans and birds of prey. Firstly, the population of vultures they would have observed would have

been rather sparse: today, each breeding pair might control a huge territory (200-400 km<sup>2</sup>; website of the Vulture Conservation Foundation, accessed 04/07/2018) and therefore the eggshell accumulation within the cave would have been caused by only one “family” of birds. The size of the family would have been small, because each female lays two eggs (but typically only one young fledges). This is interesting to note, because it implies that the accumulation of eggshell fragments would have been achieved by just two eggs per year. Secondly, recent data collected on modern bearded vulture eggs show that the surface areas can be estimated at around 1870 mm<sup>2</sup> (Hernández et al., 2018). Assuming that the minimum surface area of each fragment is ~4 mm<sup>2</sup>, as the eggshell fragments were recovered from the fraction sieved with a 2 mm mesh size, we can estimate that the maximum number of fragments from each egg would be ~465. As a consequence, the number of individual eggs effectively incorporated in the archaeological sediments would be rather low. Thirdly, most eggshell fragments come from layers without hearth features, and are in areas of the cave where the ceiling is almost or totally inaccessible to humans. These three considerations support the interpretation that the presence of eggshell in the sedimentary record represents episodes of accidental incorporation rather than exploitation of the eggs - at least not systematically.

As noted by Marín-Arroyo et al. (2009), the fact that vultures and humans cannot coexist suggests that the cave was a place of recurrent but episodic occupation, humans possibly tending to occupy the cave after the vulture young had left the nest. Bearded vultures nest in autumn, and the young would have left the cave in winter. Therefore, human occupation would have occurred during the spring/summer, at least during certain periods of the Magdalenian. However, the fact that birds and humans cannot occupy the same space at the same time does not necessarily mean that they were unaware of each other. On a practical level, we can speculate that bones processed and left over by humans could have later been eaten by the birds, although we cannot provide evidence for this, as all animal bones which may have been digested by vultures would have lost traces of the anthropogenic activity (cutmarks). Another thought-provoking aspect is the striking appearance of the bird, with its red eyes and rusty red body, the latter being achieved by the vultures rubbing themselves with iron oxides - ochre (Website of the Vulture Conservation Foundation, accessed 04/07/2018). The exceptional Magdalenian human burial, “the Red Lady”, takes its nickname (after the “Red Lady of Paviland”) from the fact that her body and/or, later, her bones had been coated in red ochre (Straus et al., 2015). The use of ochre is one of the harbingers of modern human behaviour and is frequent during the Upper Paleolithic (Brooks et al., 2018; Henshilwood et al., 2011); we are not suggesting, therefore, that the inhumation ritual was an attempt at mimicking the bird’s behaviour. Nevertheless, it is not beyond reason to speculate a multi-faceted symbolic meaning for the use of this pigment in this specific cave, which was likely inhabited by a resident breeding pair of vultures, themselves a powerful reminder of the cycle of life and death.

## **CONCLUSIONS**

This study shows that the analysis of eggshell can add another layer of information to the interpretation of human-bird relationships, including the alternate use of a site. At El Mirón, we found direct confirmation of the presence of diurnal birds of prey, previously known from only a single black vulture bone and from taphonomic alterations of the macromammal remains, from the Upper Paleolithic until the Mesolithic. This finding implies that humans and birds of prey, which are well known for occupying a special role in the life of Paleolithic people in

Cantabria (Gómez-Olivencia et al., 2018), must have found a delicate balance in this environment.

As a concluding remark, we highlight that the preservation of the eggshell proteins at this Upper Palaeolithic site from a relatively temperate environment is sufficiently good as to allow the successful identification of all fragments. However, only the availability of a large number of bird genomes, including that of the Bearded vulture, which is currently being sequenced (<https://b10k.genomics.cn/>), will be able to fully reveal the power of resolution of the technique. Therefore, we stress the importance of integrating molecular analyses with zooarchaeological and taphonomic studies of the material.

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## FIGURE LEGENDS



**Figure 1:** A) Bearded vulture (Credits: Richard Bartz, Creative Commons Attribution-Share Alike 2.5 Generic license). B) El Mirón, cave entrance; C) Plan of the excavation of El Mirón, showing the areas of recovery of the eggshell fragments (in red).

**Figure 2:** A) Example of an “Anatidae” spectrum (LOTs 14152-2, 14152-4, 14152-5, Early Upper Palaeolithic); B) Typical spectrum found for the 92 eggshell fragments attributed to Accipitridae; C) Example of a low-intensity spectrum, which shows the presence of some Accipitridae markers.

**Figure 3:** Product ion spectra for a selection of common Accipitridae markers: ALAAFIAR ( $m/z = 832.5$ ), RAEAFCQR ( $m/z = 1037.5$ ), NVWIGLYHR ( $m/z = 1158.6$ ), EEEEENVWIGLYHR ( $m/z = 1803.9$ ), GWVPFEDGCYGGFFPR ( $m/z = 1847.8$ ), EEEEEENVWIGLYHR ( $m/z = 1931.9$ ).