

Reevaluating the timing of Neanderthal disappearance in Northwest Europe

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Elucidating when Neanderthal populations disappeared from Eurasia is a key question in paleoanthropology, and Belgium is one of the key regions for studying the Middle to Upper Paleolithic transition. Previous radiocarbon dating placed the Spy Neanderthals among the latest surviving Neanderthals in Northwest Europe with reported dates as young as 23,880 ± 240 B.P. (OxA-8912). Questions were raised, however, regarding the reliability of these dates. Soil contamination and carbon-based conservation products are known to cause problems during the radiocarbon dating of bulk collagen samples. Employing a compound-specific approach that is today the most efficient in removing contamination and ancient genomic analysis, we demonstrate here that previous dates produced on Neanderthal specimens from Spy were inaccurately young by up to 10,000 y due to the presence of unremoved contamination. Our compound-specific radiocarbon dates on the Neanderthals from Spy and those from Engis and Fonds-de-Forêt demonstrate that they disappeared from Northwest Europe at 44,200 to 40,600 cal B.P. (at 95.4% probability), much earlier than previously suggested. Our data contribute significantly to refining models for Neanderthal disappearance in Europe and, more broadly, show that chronometric models regarding the appearance or disappearance of animal or hominin groups should be based only on radiocarbon dates obtained using robust pretreatment methods.

Neanderthal disappearance | Belgium | compound-specific radiocarbon dating | ancient genomic analysis

Determining when Neanderthal populations disappeared from Eurasia and anatomically modern humans (AMH) arrived is a key question in paleoanthropology (1). Recent work at more than 40 archaeological sites has established that stone tool industries linked with the Neanderthals in Europe end by ~41,000 to 39,000 calibrated years (cal) B.P. (at 95.4% probability) (2). Belgium is one of the key regions for studying the Middle to Upper Paleolithic transition. Chronological and stratigraphic evidence from several sites in this region has hinted at a gap of around 4,000 y (ca. 42 to 38 ky cal B.P.) between the Late Mousterian (Neanderthal) and the earliest dated Aurignacian (AMH) settlements (3, 4). Direct dating of Neanderthal remains, however, has produced a series of results that span this gap (5), suggesting a late-surviving Neanderthal population that could be associated with the Lincombian–Ranisian–Jerzmanowician (LRJ) (6), a so-called “transitional industry” (7). A reliable chronology is therefore crucial to test whether these Neanderthals survived as late as the dating evidence suggests. In this paper, we redate key Neanderthal specimens from Belgium using an advanced pretreatment method producing uncontaminated and reliable radiocarbon dates, allowing us to reevaluate the latest presence of Neanderthals in Northwest Europe.

Neanderthal remains have been recovered from nine caves in Belgium: La Naulette Cave, Spy Cave, Première Caverne du Bay Bonnet (Fonds-de-Forêt), Schmerling Cave (Engis), Scladina Cave, Walou Cave, Troisième Caverne de Goyet, Trou de l'Abîme, and Trou Magrite (*SI Appendix*, Fig. S1 and Table S1). Among these, Spy Cave is of particular interest because of the large number of human remains that have come from this site (description of the site in *SI Appendix*). Recent work has focused on reassessing the collection of human and faunal material excavated from the site over the last century. The original collection (excavated from 1885 to 1886) comprised 89 hominin bone fragments related to two Neanderthal individuals. Further investigations have identified more than 1,800 hominin remains, including 24 associated with the Spy Neanderthals (Spy I, Spy II, and the newly identified juvenile Spy VI) (8, 9). Seven remains of these Neanderthals have previously been directly radiocarbon dated at the

Significance

Understanding when Neanderthals disappeared is a hotly debated topic. When radiocarbon dating placed the Spy Neanderthals amongst the latest surviving in Northwest Europe, questions were raised regarding the reliability of the dates. Using a procedure more efficient in removing contamination and ancient genomic analysis, we show that previous dates produced on Neanderthal specimens from Spy are too young by up to 10,000 y. Our direct radiocarbon dates on the Neanderthals from Spy and those from Engis and Fonds-de-Forêt show a reduction of the uncertainty for the time window corresponding to Neanderthal disappearance in Northwest Europe. This population disappeared at 44,200 to 40,600 cal B.P. (at 95.4% probability). This is also earlier than previous suggestions based on dates on bulk collagen.

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The authors declare no competing interest.

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Oxford Radiocarbon Accelerator Unit (ORAU) and/or at the Centre for Isotope Research at the University of Groningen following the protocols as described in Brock et al. (10) and Dee et al. (11), respectively. Twelve accelerator mass spectrometry (AMS) dates have been obtained on collagen (SI Appendix, Table S2). They span a wide range from ~42,000 to 28,000 cal B.P. (5, 6, 9, 12). The ages for some of these specimens, therefore, fall within and after the temporal gap believed to separate the Late Mousterian and the Early Upper Paleolithic in this region (Fig. 1). Some of these specimens, dated multiple times, produced different ages, and we observed that several of these radiocarbon dates have high C:N atomic ratios (6). Caution is therefore required, because of the high probability that the radiocarbon determinations were affected by unremoved contamination.

In addition to the contamination from the sedimentary environment, ancient human bones are often treated and consolidated for better preservation, but such treatments were not systematically recorded. It is sometimes difficult to fully remove all the contamination using routine radiocarbon pretreatments, particularly when the contaminants are cross-linked to the collagen. We have optimized a more robust and chemically reliable method, which uses preparative liquid chromatography to isolate a single amino acid from the bone collagen for AMS dating (13, 14). The amino acid hydroxyproline (HYP) is the key target, since it is almost exclusively found in mammalian collagen. Extracting and dating HYP provides a level of reliability unmatched by the other methods. When re-dating Paleolithic sites using this so-called “compound-specific radiocarbon analysis” (CSRA) approach, we have observed that many of the previous radiocarbon dates obtained after less robust pretreatments are inaccurate. This can in turn lead to erroneous interpretations of human and faunal dispersal and rates of change in the archaeological, climatic, and evolutionary record (15–21).

Results

Four of the Neanderthal remains from the Spy collection were re-dated using the CSRA method, and five dates were obtained (Figs. 1 and 2 and SI Appendix, Table S2). The collagen of the vertebra fragment (Spy 737a) was previously dated at $36,250 \pm 500$ B.P. (OxA-10560). Our HYP date is $41,600 \pm 2,400$ B.P. (OxA-X-2762-6). Although the age uncertainty is larger, this date is significantly older than the previous one. This specimen was found outside of the cave and was never preserved with conservation treatments. Specimens Spy 589a and Spy 94a were discovered in the faunal material during the reassessment of the collection (22). Like Spy 737a, they have not been treated with any conservation material. Spy 589a was first dated to $33,950 \pm 550$ B.P. (OxA-21610) and $33,450 \pm 600$ B.P. (OxA-18754). The HYP measurement on the same bone produced an age of $41,700 \pm 2,300$ B.P. (OxA-38790). We obtained two dates for specimen Spy 94a: one on the third upper molar and one on the maxilla attached to the molar. The maxilla fragment was previously dated in Groningen and produced an age of $35,810 +260/-240$ B.P. (GrA-32623) (6). We obtained a HYP date that is statistically identical by virtue of its high measurement uncertainty: $37,700 \pm 2,200$ B.P. (OxA-X-2778-15). The CSRA date obtained on the tooth ($41,500 \pm 1,800$ B.P. [OxA-X-2762-21]) is, however, significantly older than the date that was previously obtained on the collagen extracted from the same tooth ($34,650 \pm 600$ B.P. [OxA-37681]). The two HYP dates obtained on the tooth (OxA-X-2762-21) and the maxilla (OxA-X-2778-15) are statistically identical. The fourth reanalyzed specimen from Spy Cave is the scapula (Spy 572a) from the original collection of human remains. It was previously dated twice in Oxford, producing ages of $23,880 \pm 240$ B.P. (OxA-8912) and $24,730 \pm 240$ B.P. (OxA-9213). It was also dated at $31,810 \pm 250$ B.P. (GrA-21546) in Groningen. These three dates appear extremely young for Neanderthals, so unremoved contamination was suspected (2, 6). We re-dated the same scapula using our CSRA

approach and obtained an age of $33,700 \pm 550$ B.P. (OxA-38356). This date is older than all previous ones obtained on this specimen, but it still appears surprisingly young compared with other dates obtained on Neanderthal specimens in Belgium and more generally in western Europe. We suspected that collagen-containing animal glue might have been used to consolidate the bone, since this preservation method was common for bones excavated in the 19th century (23, 24). The chemical composition of animal glue would be identical to endogenous collagen and therefore remain undetected during the radiocarbon dating. To explore this, we extracted DNA from Spy 572a (25), constructed libraries (26), and performed hybridization capture (27) to test for the presence of ancient hominin and other mammalian mitochondrial DNA (mtDNA). Even though we did not find evidence for the preservation of ancient hominin DNA (SI Appendix, Tables S4–S8), we found that between 9.7 and 12.8% of the isolated mtDNA fragments from Spy 572a (between 158,384 and 240,213, SI Appendix, Table S6) originated from *Bovidae* (28), whereas the remaining ones were assigned to modern human. The frequency of deamination-induced cytosine (C) to thymine (T) substitutions, which are typically elevated in authentic ancient DNA (29), were very low at the ends of both the human and bovid fragments (consistently less than 2.7%, SI Appendix, Tables S5 and S7). Moreover, we did not detect DNA from *Bovidae* in the associated extraction and library negative controls (SI Appendix, Table S6). These results indicate that the Spy 572a scapula is heavily contaminated with bovid DNA of relatively young age, pointing to preservation with glue prepared from cattle bones. We therefore conclude that the relatively young radiocarbon age of Spy 572a is an artifact of contamination with collagen from cattle, which cannot be separated from endogenous collagen prior to the radiocarbon measurements. This implies that OxA-38256 is an underestimate of the real age of the specimen and should be set to one side.

We also dated a Neanderthal lower right deciduous (or milk) second molar (LRdm2) from Engis (Fig. 3B) and the adult Neanderthal femur from Fonds-de-Forêt (Fig. 3C) using the CSRA approach (see SI Appendix for site information). We dated the tooth to $39,900 \pm 1,700$ B.P. (OxA-38394). This is significantly older than the dates previously obtained on the parietal bone from the same specimen: $26,820 \pm 340$ B.P. (OxA-8827), $27,670 \pm 140$ B.P. (OxA-17503), and $30,460 \pm 210$ B.P. (GrA-21545) (Fig. 4 and SI Appendix, Table S3). Since the femur from Fonds-de-Forêt has a very shiny surface (Fig. 3), we investigated the presence of conservation materials. Several analytical techniques can be applied to explore whether contaminants are present in bone specimens, such as Fourier transform infrared (FTIR) spectroscopy (30, 31) and pyrolysis combined with gas chromatography and mass spectrometry (Py-GC/MS) (21, 32). FTIR is rapid, but the detection of residual contamination is constrained given the detection limits of the method. Pyrolysis, however, provides information at a molecular level and can therefore be used to identify trace contaminants from the burial environment and/or derived from the conservation of the specimens. Py-GC/MS analyses revealed the presence of paraffin characterized by a series of alkanes as well as a copolymer of methyl methacrylate and butyl methacrylate (SI Appendix, Fig. S2). This shows two different and hitherto undocumented events for the conservation of the specimen. Such heavily contaminated bones can sometimes be difficult to clean with routine radiocarbon pretreatments (31, 33). The sample was prepared twice with our CSRA method and produced two statistically identical dates: $38,800 \pm 900$ B.P. (OxA-X-2767-13) and $39,500 \pm 1,100$ B.P. (OxA-38322) (Fig. 4 and SI Appendix, Table S3). The specimens from Engis and Fonds-de-Forêt both produced C:N ratios within the acceptable range for the hydroxyproline molecule, indicating that any exogenous carbon from soil contamination and conservation materials had been removed before AMS measurement. In contrast to Spy 572a, DNA analysis of the Fonds-de-Forêt femur yielded more than 152,069 hominin mtDNA fragments (SI Appendix, Table S4), 94.66% of which

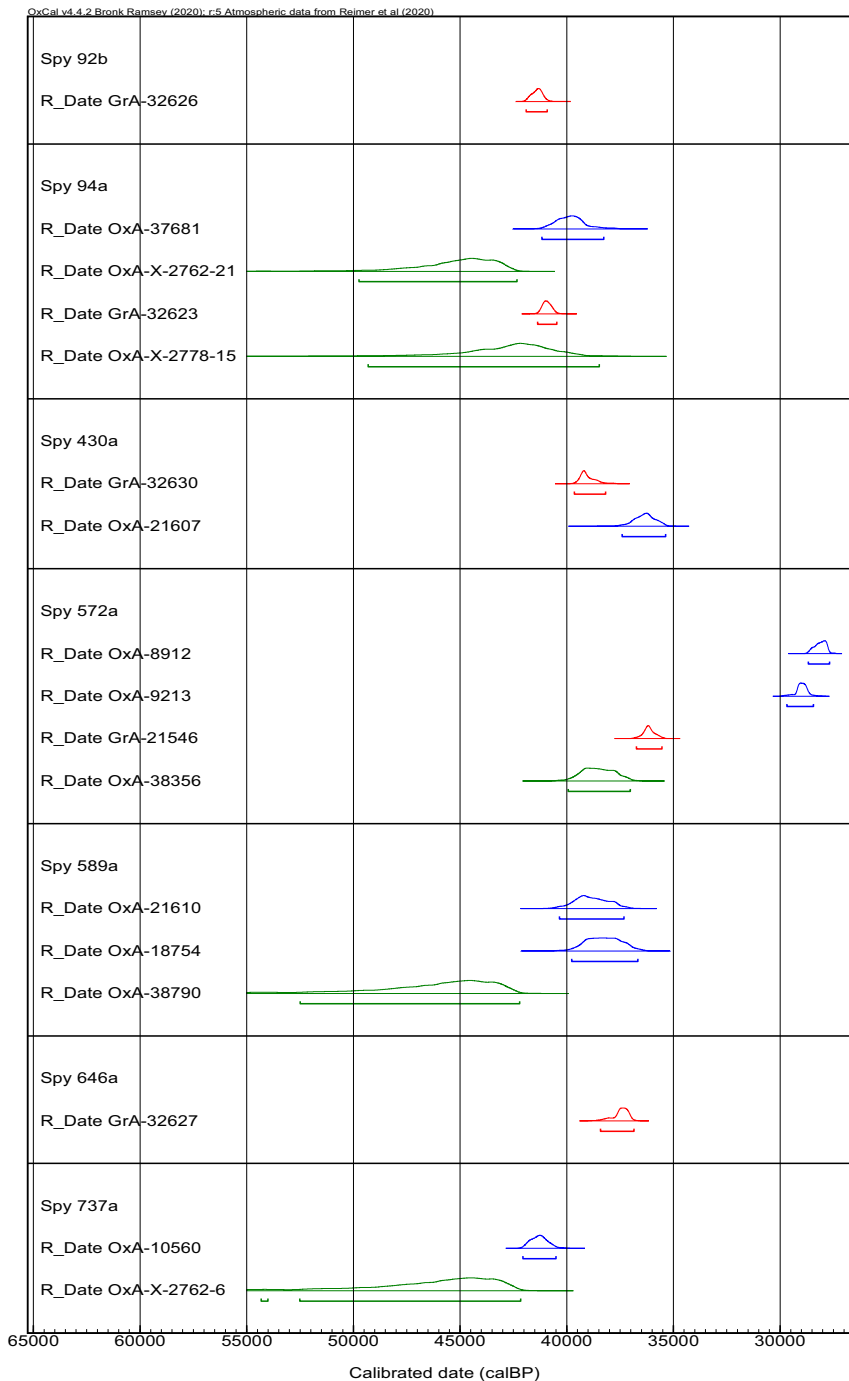


Fig. 1. Calibrated age ranges of the Neanderthal specimen from Spy Cave. Dates obtained on collagen in Groningen are reported in red. Dates obtained on collagen and hydroxyproline at the ORAU are reported in blue and green, respectively. The calibrated ages were obtained using OxCal 4.5 (87) and the IntCal20 calibration curve (88).

could be assigned to Neanderthals (*SI Appendix, Table S8*), but no DNA from animals (*SI Appendix, Table S6*). Thus, we found no indication for the presence of bone glue, which could confound compound-specific radiocarbon dating. Moreover, we reconstructed a complete mtDNA genome from the Fonds-de-Forêt femur based on 135-fold coverage in deaminated DNA fragments. This genome falls within the variation of late Neanderthals (*SI Appendix, Fig. S3*) and differs by only a single base pair (bp) from the mitochondrial genomes of Goyet Q57-1, Feldhofer 1, and Vindija 33.25.

We then used the CSRA dates to build a Bayesian model to assess probabilistically the latest Neanderthal occupation in Belgium. We built a simple single-phase model using OxCal 4.5 and the IntCal20 calibration curve to generate a probability density function (PDF) for the end boundary of the HYP determinations from Spy, Engis, and Fonds-de-Forêt Neanderthals (Fig. 5). We did not include the other six Belgian sites that produced Neanderthal specimens in the PDF because they were obviously too old, or they have not yet been directly dated, or the dates were not produced using CSRA pretreatments (see additional text in *SI*

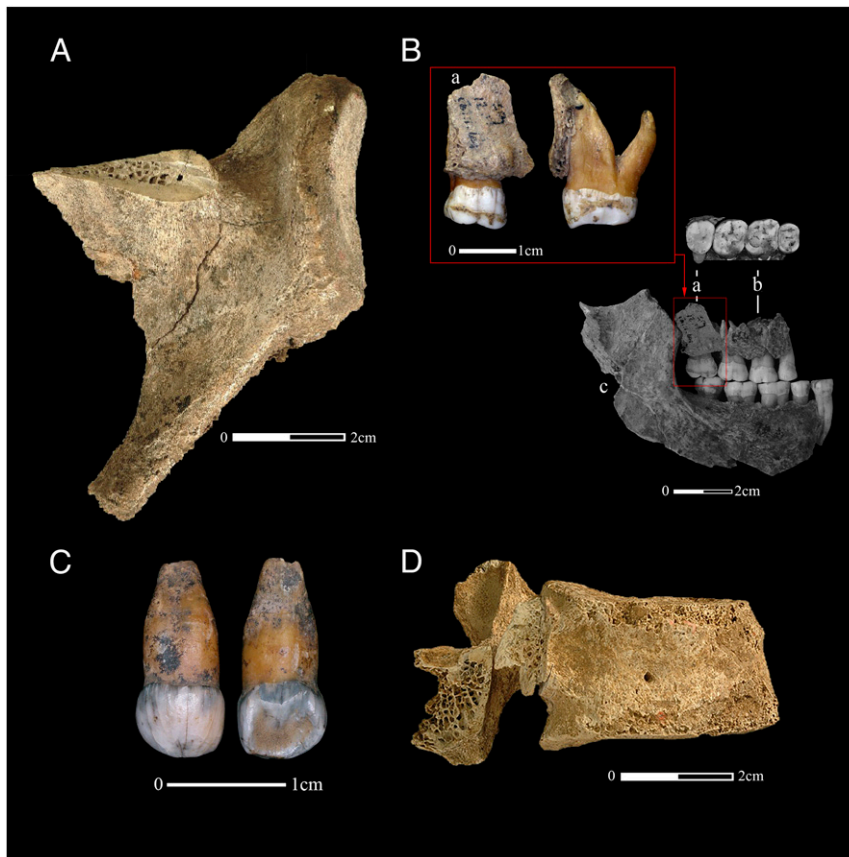


Fig. 2. The four Neanderthal specimens from Spy Cave radiocarbon dated in this study: (A) right scapula (Spy 572a, https://www.morphosource.org/concern/biological_specimens/000532268); (B, a) maxillary fragment and upper third molar (Spy 94a) associated with a maxillary fragment (B, b) (Spy 11a) and a mandibular fragment (B, c) (Spy 12a); (C) a first upper right deciduous incisor (Spy 589a); and (D) the lumbar vertebra (Spy 737a). Image by G. Abrams after pictures from M. Toussaint (A, D) and E. De Wamme (B, C; Copyright RBINS).

Appendix). By comparing this end boundary for Belgian Neanderthals with the previous Spy end boundary (2), we observe a substantial shift of the PDF toward earlier ages: 44,200 to 40,600 cal B.P. (at 95.4% probability) instead of 41,210 to 37,830 cal B.P. (at 95.4% probability).

Discussion

Our dating shows that Neanderthals disappeared from Northwest Europe much earlier than previously suggested, at 44,200 to 40,600 cal B.P. (at 95.4% probability). The potential causes of this extinction could be linked to climate change, competition, and inbreeding (34), but these are beyond the scope of this article.

It was hypothesized that the Spy Neanderthals, with three dates around 36,000 B.P., may be associated with the LRJ, a transitional technocomplex defined in northern Europe, and also discovered at this site (6, 35). This association is now less likely (at Spy Cave) because the late Neanderthal dates have been proven unreliable. It will, however, be necessary to redate the LRJ using the same CSRA approach in order to be able to discern whether Neanderthals or AMH produced the LRJ. In other regions, there is evidence that Neanderthals survived later than they did in Belgium. The Châtelperronian transitional industry from France and northern Spain, which has been linked with Neanderthals, for example, is estimated to end at 41,200 to 38,300 cal B.P. (2). Similarly, the Peștera cu Oase AMH in Romania, dated to 41,000 to 39,000 cal B.P., has recent Neanderthal introgression four to six generations prior to him (36). The earliest evidence for AMH in Europe predates 40,000 cal B.P. significantly ~45,000 cal B.P. at the Italian site of Grotta del Cavallo (37), and perhaps as early as 47,000 cal B.P. at Bacho Kiro, Bulgaria (38).

At present, a long period of overlap with AMH seems apparent. The possibility, however, remains that, because of the pervasive and influential effects of contamination on the reliability of bone dates in this period, underestimated ages are still present in the wider corpus of dates we have available to interpret. Next to sedimentary contamination, human remains are particularly prone to contamination from conservation materials, and therefore we recommend that all Paleolithic human remains should be (re)dated using CSRA approaches combined with DNA analyses to guarantee reliable results, as demonstrated here with Spy. Chronometric models regarding the appearance or disappearance of hominin groups should be based only on dates obtained using robust pretreatment methods. The crucial dates from Oase, for example, which reflect a terminus ante quem for Neanderthals in Europe based on the dates and genetic evidence, were not produced using a CSRA approach. There is a possibility that they are a minimum age. These, and other outlying dates, need to be tested first before we can be wholly confident in the wider picture of the latest Neanderthals and their spatiotemporal distribution.

Materials and Methods

Materials.

Spy Cave. In the current study, four specimens were dated and are described below (Fig. 2). They are listed after the nomenclature proposed by H. Rougier and colleagues during the reassessment of the collections (8, 22).

Spy 572a. This fragment of a right scapula (Spy 572a, Fig. 2A) belongs to the original collection and was mentioned in the first publication of the human remains and was associated with the most complete individual (39) and should now be associated with Spy II (22). The scapula presents a morphology compatible with Neanderthals (40), although part of its morphological traits can be

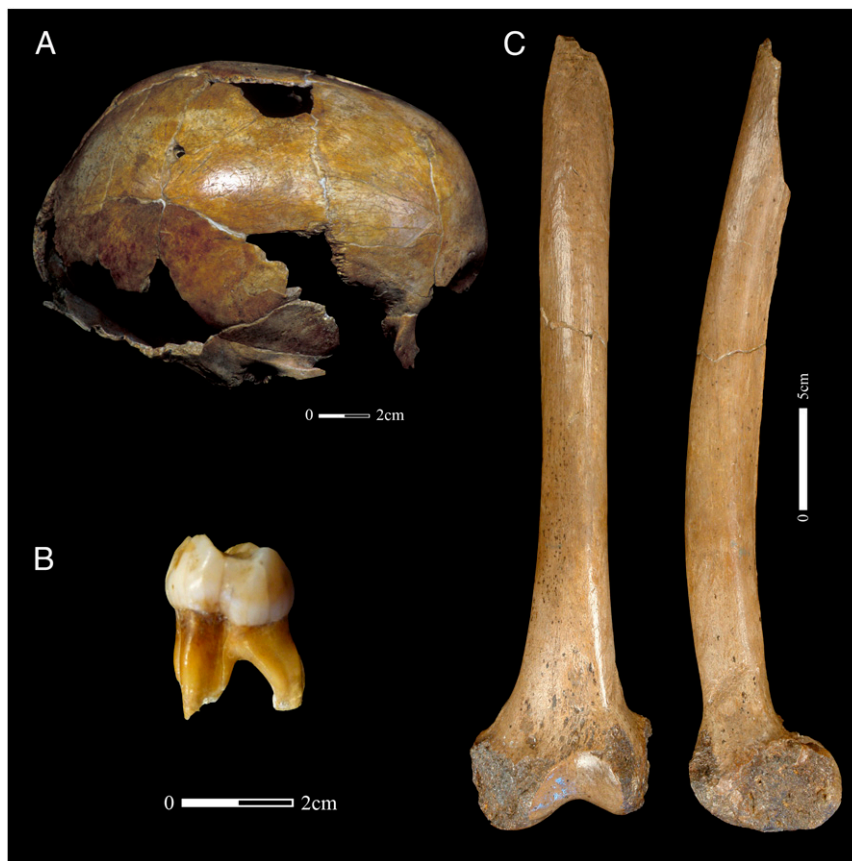


Fig. 3. The Neanderthal Child Engis 2. (A) Skullcap and (B) second lower right deciduous molar (LRdm2) radiocarbon dated in this study. Image by G. Abrams after pictures from G. Focant (A, Copyright SPW) and A. Le Cabec (B). (C) The Neanderthal left femur from Fonds-de-Forêt radiocarbon dated in this study. Image by G. Abrams modified after pictures from E. De Wamme (Copyright RBINS).

found in a minority of human remains from the Upper Paleolithic. However, none of Spy's successive excavators have discovered human remains associated with lithic tools from the Upper Paleolithic, and no radiocarbon date of human bones support such attribution (41). This specimen is part of the collections of the Department of Geology of the University of Liège. Analyses presented in this article revealed that the scapula was treated with a consolidant made of animal-derived collagen, as is probably the case with the other remains from the original collection.

Spy 94a. During the reassessment of the collections, 24 new Neanderthal remains were discovered among the faunal material, mainly in the collections of the Royal Belgian Institute of Natural Sciences (RBINS) gathered during F. Twisselmann's excavations in the 1950s (42). These excavations were carried out on the slope leading from the cave to the Orneau River, which consisted mainly of an accumulation of backfill from earlier excavations (43). From this collection, Spy 94a and Spy 589a (see below) were dated in this study. Spy 94a (Fig. 2 B, a) is an upper right third molar still attached to a small alveolar fragment of maxilla that refits with two fragments of the original collection: Spy 11a (a fragment of maxilla; Fig. 2 B, b) and Spy 12a (a fragment of mandible; Fig. 2 B, c) which were attributed first to the second individual and specifically mentioned as missing in the publication by Fraipont and Lohest (39). These remains are now attributed to Spy I (5).

Spy 589a. Spy 589a (Fig. 2C) belongs to the same collection as Spy 94a described above. It is a deciduous upper first right incisor, which, together with three other deciduous teeth (Spy 645a, Spy 592a, and Spy 594a) and a fragment of mandible (Spy 646a), is attributed to Spy VI, a third Neanderthal (immature) individual (9).

Spy 737a. A fragmentary Neanderthal lumbar vertebra (Spy 737a, Fig. 2D) was discovered in the early 2000s on the slope below Spy Cave (12). The body of the vertebra is almost complete, and the vertebral arch is well preserved. However, both transverse processes are missing, and the spinous process is very incomplete except for a small part of its root. So far, it was not possible to attribute it to a specific individual, and even the Neanderthal attribution is made difficult by the absence of derived features. The vertebra was partially destroyed to produce the ultrafiltration date OxA-10560 (12), which was compatible with

other dates made on Spy Neanderthals (6). The remaining vertebral fragment is stored in the private collection of Ph. Pirson, who found the specimen.

Even though these four specimens originate from the same site, they were discovered separately at different times. They have different taphonomic histories that can affect the collagen preservation and, therefore, the quality and reliability of the results. With the exception of the scapula fragment (Spy 572a) from the original collection (also known as De Puydt and Lohest collection), all the other remains dated here were found among the faunal material during the collection reassessment (Spy 94a and Spy 589a) or discovered by chance on the slope in front of the site (Spy 737a). The recently discovered human remains that refit with the original collection are of particular interest, since they are untreated, which helps with analyses such as radiocarbon dating and DNA analyses (6).

Schmerling Cave. The Engis 2 individual has been stored in the collections of the Department of Geology of the University of Liège since its discovery during the winter 1829 to 1830. It is composed of an almost complete calotte (skullcap, Fig. 3A), isolated teeth and a fragmentary maxilla of an infant, which is illustrated in the original publication (44). The remains of Engis 2 were located at the bottom of the cave, near an elephantidae tooth (45). The skull fell apart when Schmerling removed it from the sediments (45). However, Engis 2 is quite well preserved considering its young age [ca. 3 y old at the time of death (46)]. The calotte was first described in detail and attributed to Neanderthals by C. Fraipont, more than a century after its discovery (47), primarily using La Quina H18 as comparison. This interpretation, as well as the attribution of the isolated teeth, were later confirmed by A. M. Tillier, who had access to a larger number of comparative individuals (48). The Neanderthal attribution was also confirmed by genetic analyses (49).

The calotte was previously dated to $26,820 \pm 340$ B.P. (OxA-8827) and $30,460 \pm 210$ B.P. (GrA-21545) (12), but these dates were interpreted as too recent considering its Neanderthal attribution.

There is a real possibility, considering the antiquity of the discovery, the fragmentation that occurred during the excavation, and the shiny surface of the bone that the calotte was treated using consolidants made of animal-

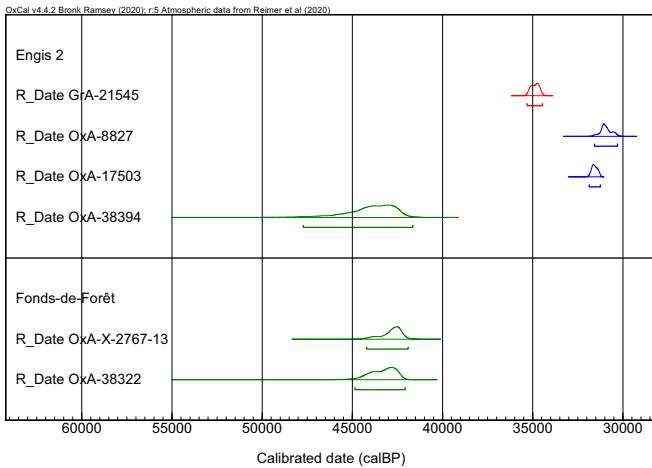


Fig. 4. Calibrated age ranges of the Neanderthal specimens from Engis and Fonds-de-Forêt caves. The calibrated ages were obtained using OxCal 4.5 (87) and the IntCal20 calibration curve (88). Dates obtained on collagen in Groningen are reported in red. Dates obtained on collagen and hydroxyproline at the ORAU are reported in blue and green, respectively.

derived collagen. Moreover, the high porosity and thinness of these bones make the extraction of uncontaminated material difficult. To avoid the risk of extracting mixed collagen (Neanderthals and modern animals), which may lead to an erroneous date, it was decided that the second lower right deciduous

molar (LRdm2), named Engis2_N°4, would be used for this study (Fig. 3B). This molar was still complete and is associated with the Neanderthal calotte (48).

Première Caverne du Bay Bonnet (Fonds-de-Forêt). Two human bones were recovered from the Première Caverne du Bay Bonnet (Fonds-de-Forêt) by F. Tihon in 1895 (50): an upper molar, which is now lost (51), and an incomplete left femur missing its proximal part (Fig. 3C). Based on its similarities to the femora from Spy, F. Tihon attributed the femur to Neanderthals. This interpretation was later confirmed by F. Twisselmann, who was the first to study the adult femur in detail (52). Tihon also proposed that the femur was broken intentionally, raising the question of cannibalism (50). However, the breakage pattern of the proximal part, testifying that it occurred when the bone was still fresh, in combination with tooth marks on the distal extremity suggest carnivore activity rather than cannibalism (53). The femur was part of the collection of the Royal Museums of Art and History before its permanent deposition in the collections of the RBINS. The shiny surface of the bone is suggestive of the presence of some conservation treatment, as is usually the case for specimens excavated in the 19th century. The femur was then sampled and sequenced for the presence of exogenous mtDNA of nonhuman origin and analyzed by pyrolysis to discern the presence of consolidants before being radiocarbon dated. These methods are described below, and the results discussed in the article.

Methods

Radiocarbon Dating. We (re)dated the Spy, Engis, and Fonds-de-Forêt Neanderthal specimens using the single amino acid radiocarbon dating method optimized at the ORAU (13). This method involves separation of the underivatized amino acids from hydrolyzed bone or tooth collagen samples using preparative Liquid Chromatography (Prep-LC). The HYP is isolated, combusted, graphitized, and AMS dated. This pretreatment approach (Coded "HYP" in the ORAU) is the most efficient technique to remove contaminants, including, but not limited to, conservation materials (unless collagen-based glue has been applied). The percent of C, percent of N, and atomic C:N ratio were measured using an automated carbon and

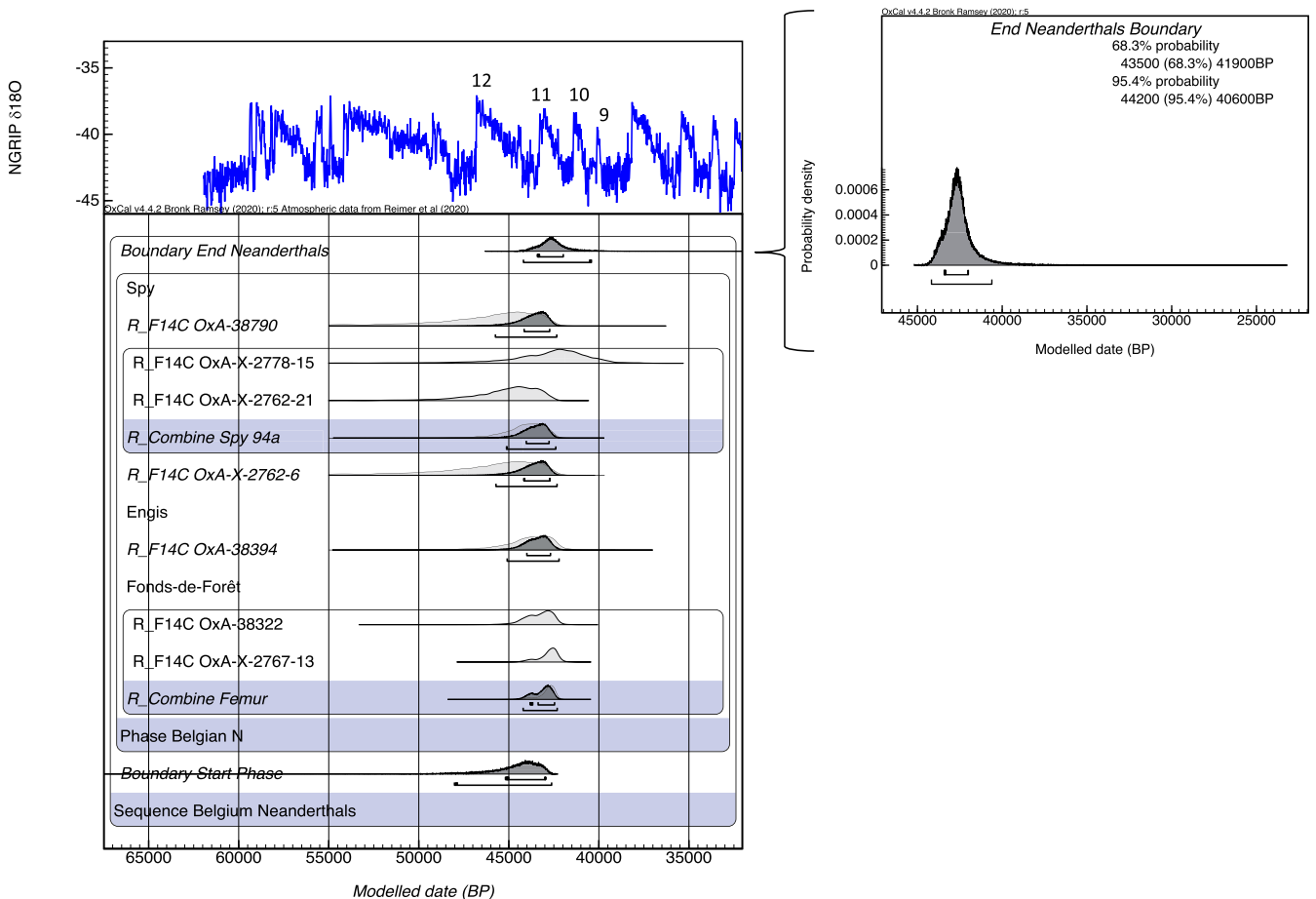


Fig. 5. Bayesian age model for Spy, Engis, and Fonds-de-Forêt caves using compound-specific determinations. The data are modeled in a single-phase model and compared tentatively against the North Greenland Ice Core Project dataset from Greenland (89). Numbers indicate interstadials in Greenland.

nitrogen elemental analyzer (Carlo Erba EA1108) coupled with a continuous-flow isotope monitoring mass spectrometer (Europa Geo 20/20).

Py-GC/MS. The Py-GC/MS analysis was performed on 450 μg of bone powder from the Fonds-de-Forêt femur and 250 μg of an uncontaminated mammoth bone sample from Latton (UK), which is used as a background standard at the ORAU. The samples were successively placed in a stainless-steel cup and inserted in the microfurnace, where they were pyrolysed at 600 °C for 20 s. The analyses were performed on an EGA/PY-3030D Micro Furnace Pyrolyzer (Frontier Lab, Japan) connected to a 6890 gas chromatograph equipped with a split/splitless injector. An HP 5MS column (30 m \times 0.25 mm, film thickness 0.25 μm , Agilent Technologies) coupled with a deactivated silica precolumn (2 m \times 0.32 mm, Agilent Technologies, USA) was used for the chromatographic separation. The program of the oven temperature used for the chromatographic separation was 40 °C isothermal for 6 min followed by a linear ramp at 20 °C/min up to 310 °C. Analyses were performed under a constant flow of helium at 1 mL/min, with a split ratio 1:20. The gas chromatograph was coupled with a 5973 Mass spectrometer (MS) (Agilent Technologies, USA). The temperature of the transfer line to the MS was 300 °C. The MS was operated in electron ionization positive mode (70 eV, scanning m/z 50 to 600). The MS ion source was kept at 230 °C and the MS quadrupole at 150 °C.

DNA Analyses.

Sampling. We removed powder samples from the Spy scapula (Spy 572a) and the femur from Fonds-de-Forêt in a clean room facility at the RBINS in Brussels using a sterile dentistry drill. We removed a thin layer of surface amounting to 15.2 mg (sample A) from an area of Spy 572a that was adjacent to where a sample was previously taken for HYP dating. An additional sample of 24.3 mg (sample B) of bone powder was taken by drilling into the cortical portion of the scapula directly underneath. A second thin layer of surface amounting to 14.7 mg (sample C) was removed from close to sampling area B. Sampling of the Fonds-de-Forêt femur was performed by extending the hole from which a sample for hydroxyproline dating had previously been taken, amounting to 26.7 mg of bone powder.

DNA extraction, library preparation, and mtDNA capture. All subsequent steps were performed at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany. DNA was extracted on an automated liquid handling platform (Bravo NGS workstation, Agilent Technologies) and as described in ref. 25. DNA extracts were converted into single-stranded DNA libraries (26) and the efficiency of library preparation was assessed using a control spike-in oligonucleotide and qPCR assays as described in (54). Libraries were amplified (55, 56) and labeled with two unique indices (57). An aliquot of each amplified library was further enriched for human mtDNA using a probe set designed based on the revised Cambridge Reference Sequence (rCRS, GenBank accession NC_01290) of the human mtDNA genome synthesized on an oligonucleotide array (27) and two consecutive rounds of on-bead hybridization capture (58). Extraction and library negative controls were carried alongside the samples throughout all steps. Enriched libraries were pooled and sequenced on an Illumina MiSeq in a double index configuration (2 \times 75 cycles) (57). Base calling was done using Bustard (Illumina).

Sequencing, data processing, and ancient DNA damage profiling. Adapters were trimmed and overlapping paired-end reads were merged into single sequences using leeHom (59). We used Burrows-Wheeler Aligner (60) with ancient DNA (aDNA) parameters ["-n 0.01 -o 2 -l 16500" (61)] to align sequences to the rCRS. Only sequences with perfect matches to the expected index combinations were retained for subsequent analyses and PCR duplicates were removed using bamrmdup (<https://github.com/mpieva/biohazard-tools>). Sequences shorter than 35 bp and with a mapping quality lower than 25 (MQ < 25) were discarded from all downstream analyses.

To investigate the presence of nonhominin mammalian DNA, we additionally aligned sequences to a set of 242 mammalian mtDNA genomes (62) and processed the alignments with a pipeline originally developed to assign mtDNA sequences from sediment samples to mammalian families (63). In brief, this pipeline uses Basic Local Alignment Search Tool (BLAST) (64) for realignments of sequences to a database of mammalian mtDNA genomes and the "lowest common ancestor" algorithm implemented in Metagenome Analyzer (MEGAN) for taxonomic assignments (65). Sequences were then evaluated separately by the family they were assigned to for the presence of elevated frequencies of terminal C to T substitutions at their ends to determine if they derived from authentic aDNA (29).

Phylogenetic inferences. To distinguish between Neanderthal, modern human, Denisovan, or Sima de los Huesos mtDNA being present in Spy 572a and the femur from Fonds-de-Forêt, we investigated the state of DNA fragments overlapping phylogenetically informative (or "diagnostic") positions (i.e., positions where the mitochondrial genomes of one of these groups differ from those of all others) (66).

We reconstructed the mitochondrial genome of the femur from Fonds-de-Forêt using mtDNA fragments longer than 35 bp that produced alignments with a mapping quality of at least 25 and that showed a C-to-T difference to the reference genome within the first and/or last three positions [deaminated fragments (67)]. We called a consensus base at each position of the mtDNA that was covered by at least three DNA fragments showing a base quality score of 20 or higher and in which at least two thirds of fragments carried an identical base (67). We converted Ts appearing in the first and last three positions of each DNA fragment in the orientation as sequenced into Ns to hinder deamination-induced substitutions affecting the calling of a consensus base.

The reconstructed mtDNA genome of the femur from Fonds-de-Forêt was aligned to the mtDNA genomes of 54 present-day humans (68), 24 Neanderthals (69–79), 4 Denisovans (28, 80, 81), a Sima de los Huesos individual (67), and a chimpanzee (82) using multiple alignment using fast Fourier transform (MAFFT) [version 7.271 (83)]. We used jModelTest2 (84) to determine the best-fitting substitution model (TrN+I+G) and generated a maximum likelihood tree using PhyML (85) with 200 bootstrap replications. All positions containing gaps and missing data were excluded from this analysis. The number of pairwise differences among the genomes was calculated using Molecular Evolutionary Genetics Analysis (MEGA) [version 7] (86).

Data Availability. All the radiocarbon data generated at the ORAU are archived internally and are also available on the laboratory's website, along with a link to the paper. The mitochondrial genome from Fonds-de-Forêt is deposited in GenBank with the accession number PRJEB39136. All other study data are included in the article and/or *SI Appendix*.

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