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Citation: Devière, Thibaut, Karavanić, Ivor, Comeskey, Daniel, Kubiak, Cara, Korlević, Petra, Hajdinjak, Mateja, Radović, Siniša, Procopio, Noemi, Buckley, Michael, Pääbo, Svante and Higham, Tom (2017) Direct dating of Neanderthal remains from the site of Vindija Cave and implications for the Middle to Upper Paleolithic transition. *Proceedings of the National Academy of Sciences*, 114 (40). pp. 10606-10611. ISSN 0027-8424

Published by: National Academy of Sciences

URL: <http://dx.doi.org/10.1073/pnas.1709235114>
<<http://dx.doi.org/10.1073/pnas.1709235114>>

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Direct dating of Neanderthal remains from the site of Vindija Cave and implications for the Middle to Upper Paleolithic transition

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Edited by Richard G. Klein, Stanford University, Stanford, CA, and approved July 28, 2017 (received for review June 5, 2017)

Previous dating of the Vi-207 and Vi-208 Neanderthal remains from Vindija Cave (Croatia) led to the suggestion that Neanderthals survived there as recently as 28,000–29,000 B.P. Subsequent dating yielded older dates, interpreted as ages of at least ~32,500 B.P. We have redated these same specimens using an approach based on the extraction of the amino acid hydroxyproline, using preparative high-performance liquid chromatography (Prep-HPLC). This method is more efficient in eliminating modern contamination in the bone collagen. The revised dates are older than 40,000 B.P., suggesting the Vindija Neanderthals did not live more recently than others across Europe, and probably predate the arrival of anatomically modern humans in Eastern Europe. We applied zooarchaeology by mass spectrometry (ZooMS) to find additional hominin remains. We identified one bone that is Neanderthal, based on its mitochondrial DNA, and dated it directly to 46,200 ± 1,500 B.P. We also attempted to date six early Upper Paleolithic bone points from stratigraphic units G₁, Fd/d+G₁ and Fd/d, Fd. One bone artifact gave a date of 29,500 ± 400 B.P., while the remainder yielded no collagen. We additionally dated animal bone samples from units G₁ and G₁–G₃. These dates suggest a co-occurrence of early Upper Paleolithic osseous artifacts, particularly split-based points, alongside the remains of Neanderthals is a result of postdepositional mixing, rather than an association between the two groups, although more work is required to show this definitively.

Vindija Cave (Croatia) | single-compound AMS dating | DNA analysis | zooarchaeology by mass spectrometry | Middle to Upper Paleolithic transition

The period between ~45,000 and 35,000 cal B.P. in Europe witnessed the so-called biocultural transition from the Middle to early Upper Paleolithic, when incoming anatomically modern humans displaced Neanderthal groups across the continent (1, 2). Significant questions still remain regarding the precise nature of this transition, the humans responsible for the various transitional early Upper Paleolithic industries, the degree of overlap between Neanderthals and modern humans, and the timing of the disappearance of the former. The European record for the transition retains its interest because it is the best-documented sequence for the disappearance of a hominin group available (3). The latest data, both radiometric and genetic, suggest Neanderthals and modern humans coexisted or overlapped for up to several thousand years in Europe until Neanderthal disappearance at around 40,000 cal B.P. (4, 5). Ascertaining the spatial attributes of Neanderthal and modern human populations in Europe is an area of active research, and a reliable chronology remains essential.

Our understanding of the biocultural processes involved in the transition have been greatly influenced by improved accelerator mass spectrometry (AMS) dating methods and their application

to directly dating the remains of late Neanderthals and early modern humans, as well as artifacts recovered from the sites they occupied. It has become clear that there have been major problems with dating reliability and accuracy across the Paleolithic in general, with studies highlighting issues with underestimation of the ages of different dated samples from previously analyzed sites (6). We have been working on redating some of the purported late-surviving Neanderthal sites from around Europe, which have included human and archaeological remains from sites such as Mezmaiskaya (Russia), where a previous directly dated Neanderthal infant yielded a radiocarbon age of ~29,000 B.P. (7), and Zafarraya (Spain), which was thought to contain Neanderthal remains clustering in age around a small group of U-series-dated animal bones between 33,400 and 28,900 B.P. (8). At Mezmaiskaya, the AMS dates obtained for the Neanderthal excavated above the previously dated individual were substantially older (9). This, along with other AMS dates from cut-marked fauna from the same archaeological horizons, suggested the original date of 29,000 B.P. could not be correct. At Zafarraya, Wood et al. (10) showed that, when redated using ultrafiltration methods, the bones that produced ages of ~33,000 B.P. were in fact beyond the radiocarbon limit, suggesting the

Significance

Radiocarbon dating of Neanderthal remains recovered from Vindija Cave (Croatia) initially revealed surprisingly recent results: 28,000–29,000 B.P. This implied the remains could represent a late-surviving, refugial Neanderthal population and suggested they could have been responsible for producing some of the early Upper Paleolithic artefacts more usually produced by anatomically modern humans. This article presents revised radiocarbon dates of the human bones from this site obtained using a more robust purification method targeting the amino acid hydroxyproline. The data show that all the Neanderthal remains are from a much earlier period (>40,000 cal B.P.). These revised dates change our interpretation of this important site and demonstrate that the Vindija Neanderthals probably did not overlap temporally with early modern humans.

Author contributions: T.D., S.P., and T.H. designed research; T.D., I.K., D.C., C.K., P.K., M.H., S.R., N.P., M.B., S.P., and T.H. performed research; T.D., D.C., and T.H. contributed new reagents/analytic tools; T.D., I.K., D.C., C.K., P.K., M.H., S.R., N.P., M.B., S.P., and T.H. analyzed data; T.D. and T.H. wrote the paper; and I.K., D.C., C.K., P.K., M.H., S.R., N.P., M.B., and S.P. contributed to writing the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1709235114/-DCSupplemental.

Neanderthal remains were unlikely to be as young as previously thought. In both cases, revised radiocarbon dates produced with more robust chemical pretreatment methods have illustrated significant underestimates in the previous dates that cannot be reconciled with a hypothesis of late-surviving refugial Neanderthals.

The Neanderthal fossil remains from level G₁ of Vindija Cave in northern Croatia have remained in the literature as potentially late individuals. Given the evidence from the Peštera cu Oase specimen, which demonstrates a recent Neanderthal ancestry in a 40,000 cal B.P. modern human from the Danube corridor (5), the renewed dating of the Vindija remains is overdue.

Two specimens, Vi-207 and Vi-208, were originally directly AMS dated in the late 1990s at the Oxford Radiocarbon Accelerator Unit (ORAU). Vi-207 is a right posterior mandible and Vi-208 is a parietal fragment, both showing Neanderthal-specific morphology (11, 12). The initial radiocarbon results were 29,080 ± 400 B.P. (OxA-8296) and 28,020 ± 360 B.P. (OxA-8295) (13). Higham et al. (14) attempted to redate these specimens by taking the very small amounts of collagen remaining from the original sample pretreatment and ultrafiltering the product before AMS dating. The revised measurements were 32,400 ± 1,800 B.P. (Vi-207: OxA-X-2089-07) and 32,400 ± 800 B.P. (Vi-208: OxA-X-2089-06), which indicated the previous dates were indeed too young. For sample Vi-208, after ultrafiltration, the C/N atomic ratio was 3.4, which indicates collagen of acceptable quality. However, for Vi-207, the >30-kDa fraction obtained produced a C/N ratio of 4.3, which indicates the presence of a high molecular weight contaminant. The radiocarbon date for this sample could therefore include a higher molecular weight noncollagenous contaminant, possibly cross-linked to the collagen. On the basis of the potential problems associated with the small size of the redated samples and the potential for remaining contaminants, OxA-X-2089-06 was considered to be a minimum age (14). If the dates are even approximately correct, however, it makes them the most recent known Neanderthals. This would imply a more extensive temporal overlap between Neanderthals and early modern humans in central Europe than has recently been documented (4).

In addition to the Neanderthal remains, level G₁ has yielded a small archaeological assemblage that contains techno-typologically Middle and Upper Paleolithic lithic artifacts plus several distinctively early Upper Paleolithic osseous points (12). It has been argued that the mix of Neanderthals, Middle Paleolithic tools, and Upper Paleolithic technology was the result of cryoturbation and *Ursus spelaeus* activity in level G₁, with elements mixing into level G₁ from both the Upper Paleolithic unit F above and the Middle Paleolithic level G₃ below (15, 16). Zilhão (17) has suggested that the G₁ lithic assemblage has parallels with the Szeletian technocomplex, and further, that there is a mixture of elements of Szeletian and Aurignacian I and II within the level [see also

Svoboda (18)]. Karvanić and Smith (19) have suggested that the mixture of elements may represent the interaction and possible acculturation between modern humans and late Neanderthals. These alternatives are testable by selecting human and organic osseous points, as well as animal bones, for renewed AMS dating. This is what we have undertaken and describe here.

Materials

A total of 10 samples from Vindija Cave were selected for AMS radiocarbon dating (Table 1). These included three previously dated Neanderthal specimens (Vi-207, Vi-208, and Vi-33.19), as well as a fourth Neanderthal bone (Vi-*28) discovered using zooarchaeology by mass spectrometry (ZooMS) screening. In addition, to test the reality of the co-occurrence of earlier Upper Paleolithic bone and antler point artifacts with the Neanderthal remains, we selected six osseous points for dating (SI Appendix, Fig. S1). To test collagen preservation, we took ~3–5 mg bone powder, using tungsten carbide drills, and measured the %N content. This is an indicator of collagen preservation (20). The results show that only one bone point sample (Vi-3446) had sufficient levels of nitrogen to warrant full sampling for collagen extraction and AMS dating; the remainder failed and therefore were not sampled further (SI Appendix, Table S1). We also included the sample from a split-based bone point (Vi-3437) that had been analyzed in the laboratory previously, producing only a small collagen yield. We decided to attempt to redate it, using a larger starting mass of bone powder. Unfortunately, there was insufficient collagen remaining from this sample after pretreatment. We also selected four animal bones of *Cervidae* (Vi-*17), *Panthera* sp. (Vi-*6), *Ursus* sp. (Vi-*7), and *Bovidae* (Vi-*60), identified by ZooMS from stratigraphic units G₁ and G₁–G₃ to explore the potential issue of postdepositional mixing in the deposits further.

Results and Discussion

ZooMS Collagen Fingerprinting. We used ZooMS to identify potential hominin bone fragments among the unidentified faunal remains from the G₁ and G₃ levels, as well as the stratigraphic unit G₁–G₃. The majority of the 383 samples we analyzed yielded poor collagen preservation, which prevented any identification to genus or taxon. Only 101 samples produced identifiable spectra; a summary of all taxa identified by ZooMS is shown in SI Appendix, Table S2. This assemblage is dominated by *Ursus*, and only six of the 27 taxa identified by morphological study of the bones in Miracle et al. (21) could be identified here. We identified a single hominin specimen (Fig. 1 A and B), which again highlights the use of applying such techniques to groups of unidentified Paleolithic bone samples. The bone was analyzed using ancient DNA techniques to enable a formal species identification.

DNA Analysis of the Human Bones. Genomic analysis based on mitochondrial DNA revealed that all four human specimens fall into Neanderthal mitochondrial variation. Full mitochondrial genomes of Vi-207 and Vi-*28 were reconstructed with an average coverage of 103-fold and 257-fold, respectively. The mitochondrial DNA sequence of Vi-207 was identical to Vi-33.25 and Feldhofer 1 mitochondrial genomes, whereas Vi-*28 had an identical mitochondrial

Table 1. Samples selected for AMS dating

ORAU P Number	Sample Reference	Archeology Level	Species
39039	Vi-33.19 (SP2756)	G ₃	Neanderthal
41681	Vi-*28 (SP4162)	G ₁	Neanderthal
41415	Vi-208/Vi-11.29 (SP3563)	G ₁	Neanderthal
41416	Vi-207/Vi-11.41 (SP3562)	G ₁	Neanderthal
41417	Vi-3446	Fd/d+G1	Not known (point)
41687	Vi-3437	G ₁	Not known (split-based point)
41803	Vi-*17	G ₁	<i>Cervidae</i>
41801	Vi-*6	G ₁	<i>Panthera</i> sp
41802	Vi-*7	G ₁	<i>Ursus</i> sp
41804	Vi-*60	G ₁ –G ₃	<i>Bovidae</i>

All samples produced enough collagen for radiocarbon dating with the exception of P41687, which failed to produce any collagen. See SI Appendix, Fig. S1 for images of the bone points included in this study.

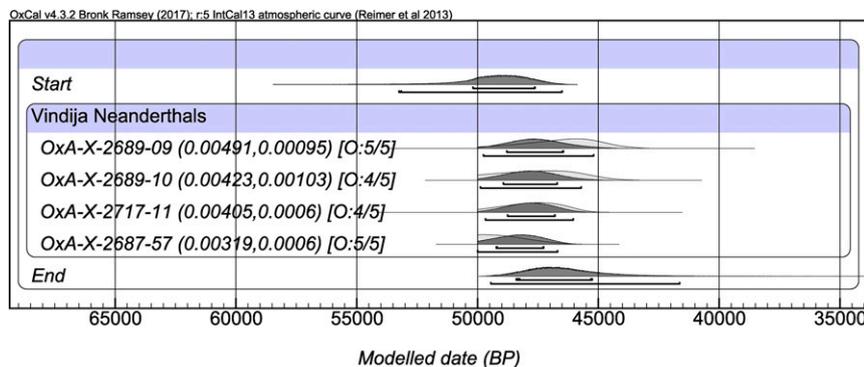


Fig. 2. Bayesian age model showing the calibrated HYP ages of the four Neanderthal samples from Vindija Cave. The model is a simple phase model in OxCal 4.3 (47), in which all F14C determinations are assumed to have no relative order. “Start” and “End” correspond to the boundaries calculated by the model. The calibration curve of Reimer et al. (48) was used to calibrate the results. Details can be found in the [SI Appendix](#).

noncollagenous high molecular weight contaminants, probably crosslinked to the collagen, were still present in the sample previously dated. It is only by hydrolyzing the collagen and selecting the hydroxyproline that we were able to successfully remove these contaminants.

The AMS measurement of the third human bone from level G_1 (Vi-*28), identified using the ZooMS method, gave a date of $46,200 \pm 1,500$ B.P. For these three HYP (extraction of hydroxyproline from hydrolyzed bone collagen) dates obtained on the Neanderthal bones from level G_1 , we performed a χ^2 test using the modern fraction F14C and its error. The error weighted mean is 0.0038 ± 0.0005 with a t value of 2.57. If t is <5.99 , the value for χ^2 , the error weighted mean is not significant. This shows that the results obtained on the three Neanderthal bones from level G_1 are statistically in agreement. Vi-33.19 (level G_3) was also dated using the hydroxyproline method and produced a date of $44,300 \pm 1,200$ B.P., which is a more precise date than the one obtained using the AF procedure ($45,300 \pm 2,300$ B.P.). The AF determination had a low target current in the AMS (85% of normal current) and was imprecise for this reason. The fact that the two dates overlap suggests no significant contamination in this bone. Taken together, these dates show a significantly older occupation of the site by Neanderthals, suggesting the site cannot be considered to be a refugium for late-surviving Neanderthals (Fig. 2, code in [SI Appendix](#)). The two other dates obtained on samples Vi-33.26 and Vi-75, even if obtained with a method that can be less efficient in removing contamination, confirm the dating of level G is more than 42,000 B.P.

Only one bone point (Vi-3446-P41417) produced enough nitrogen during prescreening to warrant pretreatment chemistry. Less than 500 mg bone was available, so we applied an AG (gelatin) treatment to maximize the collagen yield. We obtained a date of $29,500 \pm 400$ B.P., which we consider to be a minimum age, as this pretreatment has been shown before to underestimate the age of ancient bones such as this (Table 3). To test possible perturbation in level G_1 , we also dated four faunal samples identified as originating from levels G_1 and G_1 – G_3 , using

ultrafiltration (AF). These samples produced dates that were all greater than or very close to the radiocarbon limit (Table 3). These samples are less likely to have been treated with conservation materials than the human bones, and therefore were not prepared using the HPLC method. Three of these faunal bones are from level G_1 (Table 1). We see more variation that would be expected statistically if we combine the three Neanderthal bones and three faunal bones from level G_1 for a χ^2 test (error weighted mean, 0.0027 ± 0.0004 ; $t = 22.52$; $\chi^2 = 11.07$). This shows there is bone of variable radiocarbon age in the G_1 level and suggests the possibility of postdepositional mixing and movement of material. In consequence, the bone tools cannot be associated with the Neanderthal remains unless further direct radiocarbon dating using the HYP approach is undertaken. Clearly, the low collagen content of the points appears to preclude this at this time.

Conclusions

Single-amino acid AMS dating of the Vindija Neanderthals has yielded results that are substantially older than the previous ages that were initially obtained. We have shown that the Neanderthals predate $\sim 44,000$ cal B.P. The results suggest this group was not a late-surviving refugial Neanderthal population, as previously thought, and means the group almost certainly did not overlap with early anatomically modern humans in this part of Europe. Despite our best attempts, we were not able to date the bone industry associated with the archaeology of level G_1 . The one date we obtained from a later stratigraphic unit was younger than 30,000 B.P., but because the bone was not treated with the most rigorous pretreatment chemistry methods, it could potentially be older. The dating of other faunal materials from level G_1 highlighted a significant range in age, which could indicate a perturbation of the general sequence. The question, then, of whether some of the points could have been produced by Neanderthals remains open; however, it is parsimonious to conclude that the split-based point at least must have a maximum age of 32,000–34,000 B.P. based on evidence for its association with the Aurignacian in other regions, and so it likely postdates the Vindija Neanderthals significantly.

Table 3. Radiocarbon determinations and analytical data from Vindija faunal remains and bone point

OxA/OxA-X	CRA	\pm	PCode	Species	Used, mg	Yield, mg	%Yield	%C	$\delta^{13}\text{C}$, ‰	$\delta^{15}\text{N}$, ‰	C/N
34458	29,500	400	AG	Not known (bone point)	450	6.58	1.5	46.0	−19.4	3.5	3.4
2695-21	>46,700		AF	<i>Cervidae</i>	960	2.60	0.3	35.9	−20.7	5.6	3.3
34471	>48,400		AF	<i>Panthera</i> sp.	1,100	9.09	0.8	39.1	−18.9	10.1	3.2
34472	>49,000		AF	<i>Ursus</i> sp.	1,000	4.53	0.5	37.1	−23.0	8.8	3.2
34473	47,200	2,900	AF	<i>Bovidae</i>	950	40.41	4.3	42.2	−20.8	4.4	3.2

See [SI Appendix](#), [Table S2](#) caption for details of the terms used.

Bone points have been recovered throughout Eurasia with dates as early as ~37,000 B.P. (e.g., from the Aurignacian site of Pes'ko and the Châtelperronian site of Arcy-sur-Cure) (6, 25, 26). It is therefore clear that both anatomically modern humans and Neanderthals produced bone points, with only split-based bone/antler points being diagnostic of the earlier facies of the former.

Our perception of the biological transition between Neanderthals and modern humans has changed radically during the last decade. Evidence suggests interbreeding and a significant temporal overlap between the two from ~44,000–40,000 cal B.P. On the basis of our hydroxyproline dates and the DNA results, the Vindija Neanderthals date before the period when the first modern humans arrived into Europe and interbred with Neanderthals.

Methods

Bone Screening by ZooMS. We used collagen peptide mass fingerprinting or ZooMS to analyze preserved type 1 collagen (COL1) from small unidentified bone fragments from the Vindija site. COL1 is characterized by three polypeptide (alpha) chains, and within these chains there are small differences in the amino acid sequences among different species. These offer the possibility of identification of species-specific amino acid markers. We used matrix-assisted laser desorption/ionization mass spectrometry to determine the relative mass-to-charge (m/z) values of the tryptic digest peptides in each bone sample. By comparing these values with a library of known fauna, we identified bones to genus or species in some instances (27–31). We screened a total of 383 unidentified bone samples from Vindija Cave (levels G1, G3, and stratigraphic unit G1–G3).

Radiocarbon Dating. We used two different methods to prepare the samples for AMS dating. First, samples from artifacts were pretreated following the routine ORAU procedure, comprising a decalcification, base wash, reacidification, gelatinization, and ultrafiltration (coded AF in the ORAU), as described by Brock et al. (20). Sample P41417 produced too little collagen for ultrafiltration, so the treatment was halted after gelatinization (coded AG). The four human bone samples were dated using the single amino acid radiocarbon dating method developed at ORAU (32). This method involves separation of the underivatized amino acids from hydrolyzed bone collagen samples using preparative HPLC. Details of the method are described in the *SI Appendix*. Hydroxyproline (HYP), was isolated by Prep-HPLC, combusted, graphitized, and AMS-dated. This pretreatment approach is the most efficient technique to remove contaminants, including conservation materials (unless collagen-based glue has been applied).

DNA Analysis.

DNA extraction and library preparation. Genomic analysis of the four human bones was performed at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany. Three samples (Vi-207, Vi-208, and Vi-*28) were

prepared specifically for this project, whereas Vi-33.19 was previously analyzed as part of a separate study (22). Bones were sampled using a sterile dentistry drill, and between 15 and 30 mg bone powder was used for DNA extraction, with the exception of Vi-*28, in which three separate parts of the bone were sampled (*SI Appendix, Table S3*). DNA extraction was performed using a method for ultrashort DNA fragment retrieval (33) with modifications (34). The bone powder of Vi-207 and Vi-208 was treated with 0.5% hypochlorite solution before DNA extraction (34) to remove microbial and modern human DNA contamination. Next-generation sequencing libraries were prepared from 10 μ L of each extract, using two single-stranded library preparation schemes: Vi-207 and Vi-208 with a previously published method (35), with modifications from Korlević et al. (34), and Vi-*28 with an automated library preparation scheme (36). Libraries were amplified and labeled with a pair of unique index sequences (37, 38).

Enrichment of the mitochondrial DNA and sequencing. Amplified libraries were enriched for human mitochondrial DNA, using a bead-based hybridization capture (39) with modifications (40). Enriched libraries were sequenced on an Illumina MiSeq platform with a double index configuration (41). Overlapping paired-end reads were merged (42) and mapped to the revised Cambridge Reference Sequence mitochondrial genome (rCRS; NC_012092), using Burrows-Wheeler Aligner (43) with parameters “-n 0.01 -o 2 -l 16500” (44). Analyses were restricted to sequences with perfect index combinations. PCR duplicates were removed using bam-rmdup (<https://bitbucket.org/ustenzel/biohazard-tools>), and mapped sequences longer than 35 bases with a mapping quality of at least 25 were retained for subsequent analyses. To assess whether the results could be affected by present-day modern human contamination, sequences with ancient DNA specific cytosine to thymine substitutions (C to T) at the 5' or 3' molecule end were selected and analyzed separately.

Phylogenetic analyses. To reconstruct the mitochondrial genomes of the three previously unanalyzed hominin samples, DNA sequences were realigned to the Vindija 33.16 mitochondrial genome (AM948965) (45). Data were processed as described earlier. Data analysis was performed as previously published (ref. 28 for full mitochondrial genomes, ref. 46 for partial mitochondrial data).

ACKNOWLEDGMENTS. We thank Fred H. Smith (Illinois State University), Jadranka Mauch Lenardić (Institute for Quaternary Palaeontology and Geology, Croatian Academy of Sciences and Arts), and Pavao Rudan (Croatian Academy of Sciences and Arts) for their assistance in sampling and analyzing the Neanderthal and faunal bone from Vindija Cave. Ian Cartwright (Institute of Archaeology, University of Oxford) took the photographs of the Vi-*28 Neanderthal bone. Rachel Hopkins ran the %N tests on some of the bones. We also thank the staff of the Oxford Radiocarbon Accelerator Unit at the University of Oxford and the Department of Evolutionary Genetics at the Max Planck Institute for Evolutionary Anthropology, Leipzig, for their support. The research leading to these results has received funding from the European Research Council (ERC) under the European Union's Seventh Framework Programme (FP7/2007-2013); ERC Grant 324139 “PalaeoChron” (to T.H.) and ERC Grant 694707 “100 Archaic Genomes” (to S.P.). We also acknowledge the support of the Royal Society for funding a University Research Fellowship (to M.B.).

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