

Clusters of iron-rich cells in the upper beak of pigeons are macrophages not magnetosensitive neurons

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Understanding the molecular and cellular mechanisms that mediate magnetosensation in vertebrates is a formidable scientific problem^{1,2}. One hypothesis is that magnetic information is transduced into neuronal impulses by using a magnetite-based magnetoreceptor^{3,4}. Previous studies claim to have identified a magnetic sense system in the pigeon, common to avian species, which consists of magnetite-containing trigeminal afferents located at six specific loci in the rostral subepidermis of the beak^{5–8}. These studies have been widely accepted in the field and heavily relied upon by both behavioural biologists and physicists^{9–11}. Here we show that clusters of iron-rich cells in the rostro-medial upper beak of the pigeon *Columbia livia* are macrophages, not magnetosensitive neurons. Our systematic characterization of the pigeon upper beak identified iron-rich cells in the stratum laxum of the subepidermis, the basal region of the respiratory epithelium and the apex of feather follicles. Using a three-dimensional blueprint of the pigeon beak created by magnetic resonance imaging and computed tomography, we mapped the location of iron-rich cells, revealing unexpected variation in their distribution and number—an observation that is inconsistent with a role in magnetic sensation. Ultrastructure analysis of these cells, which are not unique to the beak, showed that their subcellular architecture includes ferritin-like granules, siderosomes, haemosiderin and filopodia, characteristics of iron-rich macrophages. Our conclusion that these cells are macrophages and not magnetosensitive neurons is supported by immunohistological studies showing co-localization with the antigen-presenting molecule major histocompatibility complex class II. Our work necessitates a renewed search for the true magnetite-dependent magnetoreceptor in birds.

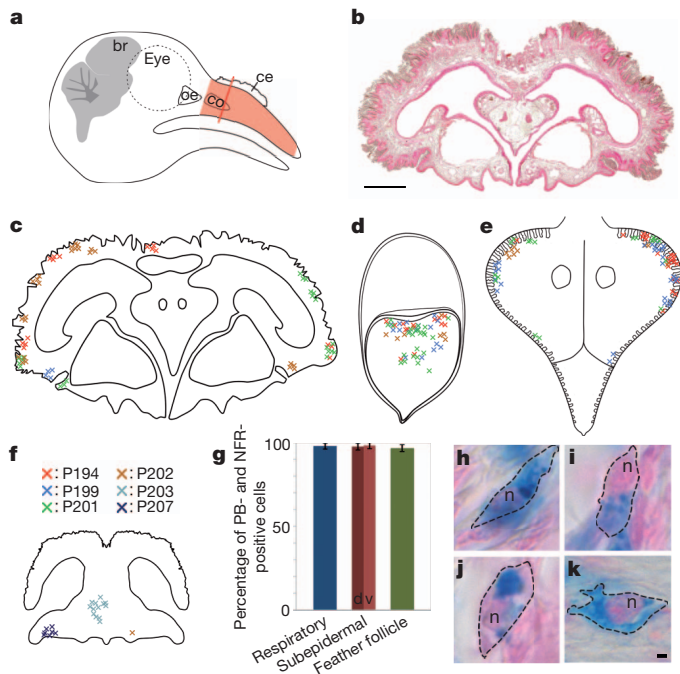
Each year millions of birds complete lengthy journeys guided by the Earth's magnetic field. Current evidence indicates that the detection of magnetic fields is mediated by an inclination-sensitive light-dependent compass that resides in the retina^{12,13}, and an intensity-sensitive apparatus that is believed to provide information about the magnetic map, is associated with the trigeminal nerve, and is thought to rely on biogenic magnetite (Fe₃O₄)¹⁴. The trigeminal nerve was first implicated in magnetoreception in the bobolink *Dolichonyx oryzivorus*, and was suggested to be sensitive to small alterations in magnetic stimuli¹⁵. Subsequent studies revealed that the ophthalmic branch is required for pigeons to perform an intensity-based conditioning task³, and that neurons in the trigeminal brainstem complex of European robins are activated when the birds are subjected to non-uniform magnetic fields¹⁶. These data have led to the proposition that the sensory cells responsible for magnetite-based magnetoreception lie in the upper beak of birds^{3,17}. Previous studies^{5,7} have claimed that clusters of iron-containing neurons in six specific bilateral locations in the rostral dermis of the upper beak of pigeons constitute a magnetic sense system⁷. It has been contended that this system consists of unmyelinated dendrites that contain superparamagnetic spherules surrounded by iron platelets that are composed of magnetite and

maghemite, and that the system is a common sensory apparatus in birds^{5–7}. These assertions have formed the basis for a host of behavioural studies and theoretical calculations that aim to advance the magnetite theory of magnetoreception^{9,10,18–20}.

To investigate this putative magnetic sense system, we undertook a systematic analysis of the prevalence and distribution of all iron-rich cells in the upper beak of the pigeon. We perfused adult pigeons (Nuremberg cohort, $n = 12$), and sectioned the upper beak from the caudal respiratory concha to the tip of the beak in the coronal plane (Fig. 1a, b). We stained serial sections (10 μm) with Prussian blue (PB) to label ferric iron, and nuclear fast red (NFR) to identify nuclei, followed by counting of all PB-positive cells. We consistently observed PB-positive cells in three specific regions: (1) in the stratum laxum of the dorsal and/or ventral subepidermis; (2) in the buds of feather follicles; and (3) in the basal region of the respiratory epithelium (Fig. 1c–e). We confirmed this pattern of staining in a larger collection of pigeons originating from seven different lofts ($n = 172$). PB-positive cells in all three regions were characterized by the presence of multiple dark blue spherules (0.25–5.0 μm in size) and/or by light blue cytoplasmic staining with a notable nucleus (Fig. 1g–k and Supplementary Figs 1–3). Subepidermal PB-positive cells in caudal and medial regions were predominantly found in the dorsal subepidermis (Fig. 1c), whereas those PB-positive cells located rostrally were found in the ventral subepidermis lining the inner roof of the beak (Fig. 1f and Supplementary Fig. 1). PB-positive cells in the feather follicle clustered in the apical region of the bud (Fig. 1d and Supplementary Fig. 2), and those in the respiratory epithelium were predominantly found within the lateral edges of the concha (Fig. 1e and Supplementary Fig. 3).

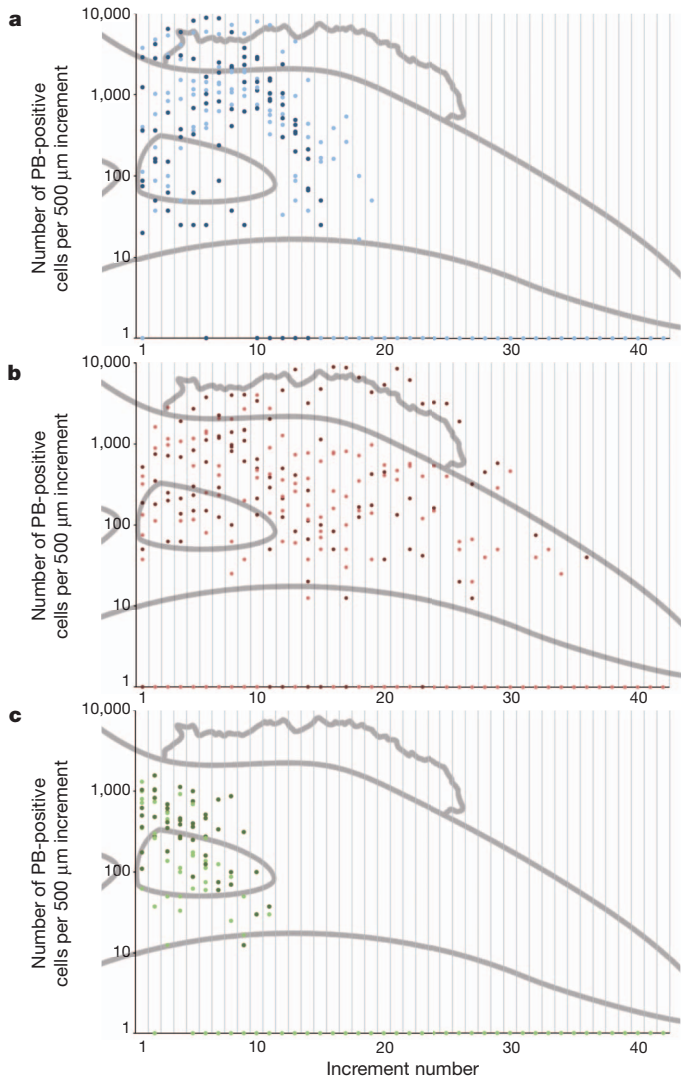
As it is believed that iron-rich cells in the upper pigeon beak are limited to six discrete bilateral anatomical loci⁵, we mapped the distribution of PB-positive cells along the rostro-caudal axis of the beak. To do this accurately we first created a three-dimensional topographic map of the pigeon beak by undertaking high-resolution magnetic resonance imaging (MRI) coupled with micro-computed tomography (micro-CT) scanning, identifying four specific anatomical landmarks (Supplementary Movies 1, 2 and Supplementary Figs 4, 5). After staining serial sections, we counted PB-positive cells and used our landmarks to map the distribution of cells along the rostro-caudal axis ($n = 12$). We found that PB-positive cells in the respiratory epithelium and feather follicles were restricted to caudal regions, whereas those in the subepidermal region were found in clusters along the length of the beak with no apparent bilateralization (Fig. 2a–c and Supplementary Figs 6, 7 and Supplementary Table 1). We found no significant differences in the total number of PB-positive cells between sexes (respiratory epithelium ($P > 0.5$), subepidermis ($P > 0.1$), feather follicle ($P > 0.1$)) (Supplementary Fig. 8), but observed an extremely large variation in the number and distribution of PB-positive cells when comparing birds of the same age and sex. For instance, pigeon 200 had ~200 PB-positive cells in the subepidermis, whereas pigeon

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203 had $\sim 108,800$ PB-positive cells located in numerous clusters along the length of the beak. Although our serial quantification samples a section every 120 μ m we did not find the six 350- μ m-long bilateral clusters that are claimed to constitute a magnetic sense system⁷. We speculated that our pigeon strain might harbour a large genomic deletion that accounts for the absence of this putative magnetosensitive system. To investigate this we quantified PB-positive cells in pigeons from another loft (Vienna cohort, $n = 6$). Similar to our Nuremberg cohort we did not find six bilateral clusters, and once again observed a large variation in the distribution and number of PB-positive cells (Supplementary Fig. 9 and Supplementary Table 2). This variation is not consistent with a genetically encoded sensory apparatus responsible for magnetosensation.

Next we asked whether PB-positive cells are neurons by triple staining sections with PB, NFR, and one of three different antibodies that label neuronal structures: neurofilament (NF), TUBB3 and MAP1B ($n \geq 5$ birds). In the respiratory epithelium we observed 0.04% co-localization with NF ($n = 1,208$ cells), 0.6% co-localization with TUBB3 ($n = 2,818$ cells), and 0.01% co-localization with MAP1B ($n = 2,213$ cells). In the subepidermis we found no co-localization with NF ($n = 471$ cells) or MAP1B ($n = 803$ cells), and only 0.06% co-localization with TUBB3 ($n = 1,309$ cells). Finally, in the feather follicle we found no co-localization with NF ($n = 286$ cells) or MAP1B ($n = 295$ cells), and only 0.24% co-localization with TUBB3 ($n = 407$ cells) (Supplementary Figs 10, 11). The simplest explanation for the



very small amount of apparent co-localization we observed is that two cells, one PB positive and the other positive for a neuronal marker, lie in the same vertical plane, and because of the nature of the chemical stain used cannot be distinguished from one another. Taken together, our results strongly suggest that the clusters of PB-positive cells in the beak of the pigeon are not neurons.

To ascertain the true identity of the PB-positive cells, we undertook an analysis of their ultrastructure using transmission electron microscopy (TEM) ($n = 3$ birds) (Supplementary Fig. 12). We observed ferritin-like granules (6–9 nm) throughout the cytoplasm of PB-positive cells from all regions and in some instances haemosiderin masses and/or membrane-bound electron-dense organelles known as siderosomes^{21,22} (~ 300 nm) (Fig. 3a–f and Supplementary Fig. 13). Energy-filtered transmission electron microscopy (EFTEM) confirmed that each electron-dense granule was composed of iron (Supplementary

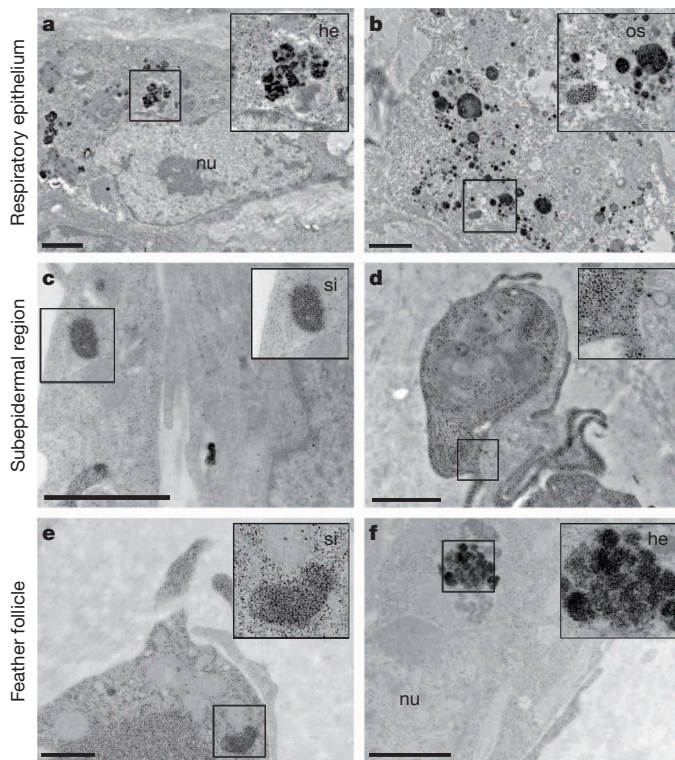


Figure 3 | Ultrastructure of PB-positive cells. a–f, Representative electron micrographs of two different PB-positive cells in the respiratory epithelium (a, b), subepidermal region (c, d) and the feather follicle (e, f; $n = 3$ birds). Cells in all regions were found to contain ferritin-like granules (6–9 nm in diameter) that are present throughout the cytoplasm. In addition we observed haemosiderin (he) clumps and/or membrane bound siderosomes (si). Osmophilic lipid droplets (os) are visible in cells in the respiratory epithelium in b. Cells in the subepidermal region and feather follicle were noted for their slender cytoplasmic projections resembling filopodia, that are seen to engulf a cell in d. Cells in all regions were nucleated (nu). See also Supplementary Fig. 13. Scale bars, 1 μ m.

Fig. 14). Selected area electron diffraction (SAED) failed to identify any cellular structures that contained magnetite, but showed that haemosiderin masses in the feather follicle consist of a goethite-like material ($n = 3$ birds), whereas siderosomes in the respiratory epithelium are comprised of ferrihydrite ($n = 2$ birds) (Supplementary Fig. 15 and Supplementary Table 3). On a cellular level, PB-positive cells in the respiratory epithelium are characterized by the presence of osmophilic lipid vacuoles (Fig. 3b), whereas those cells originating from the feather follicle and subepidermal region had notable dendritic extensions that resembled filopodia (Supplementary Fig. 13d–i, k). In some instances these cytoplasmic tentacles appeared to engulf neighbouring cells, suggesting to us that the PB cells may be phagocytic macrophages (Fig. 3d).

Macrophages are known to reside in the spleen, dermis and respiratory mucosa of multiple species, and to have a vital role in host defence and iron homeostasis²³. Iron accumulates within macrophages during the catabolism of haemoglobin and is stored as ferritin²⁴. In one class of macrophages known as siderophages, ferritin accumulates in membrane-bound siderosomes, which can then undergo proteolytic processing forming haemosiderin. This accumulation of iron renders these cells PB positive^{25,26}. To ascertain whether the PB-positive cells in the upper beak of the pigeon were siderophages, we stained cryosections with sera against major histocompatibility complex class II (MHC II), which labels antigen-presenting cells including macrophages, alongside positive and negative controls ($n \geq 4$ birds) (Fig. 4a–c and Supplementary Fig. 11)²⁷. We observed MHC II co-localization with 98.8% of PB-positive cells in the respiratory epithelium ($n = 104$ cells),

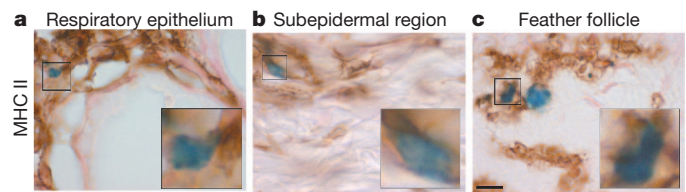


Figure 4 | MHC II immunohistochemistry on PB-positive cells.

a–c, Representative images of coronal sections triple stained with PB, NFR, and sera against the antigen-presenting marker MHC II ($n \geq 4$ birds). MHC II staining was predominant on the surface of cells and found to co-localize with $\geq 94\%$ of PB-positive cells in all regions. Controls are shown in Supplementary Fig. 11. Scale bar, 10 μ m.

95% of PB-positive cells in the subepidermis ($n = 92$ cells), and 94.4% in the feather follicle ($n = 205$ cells). Taken together with our anatomical mapping, subcellular data and neuronal staining, we conclude that clusters of PB-positive cells in the upper beak of the pigeon *C. livia* are macrophages, not magnetosensitive neurons.

As macrophages are not unique to the upper beak, our finding predicts that PB-positive cells should be found throughout the pigeon. To test this we stained skin samples from the back, abdomen, neck, scalp, wing and lower beak of the pigeon ($n \geq 3$ birds). This revealed widespread PB-positive staining in the subepidermis and feather follicle, which was indistinguishable from that observed in the upper beak (Supplementary Fig. 16). Our conclusion further predicts the infiltration of PB-positive macrophages in response to tissue damage or host invasion. We observed such a response in the beak of one of our pigeons (P199), where a large inflammatory lesion with a necrotic centre was surrounded by lymphoplasmacytic cells, including $\sim 80,000$ PB-positive cells that were again characterized by constellations of blue spherules and/or by light blue cytoplasmic staining (Supplementary Fig. 17).

Although we cannot exclude the possibility that a small number of sparsely distributed magnetoreceptors reside at an unknown location in the upper beak of pigeons, this study finds no evidence to support the existence of a subepidermal magnetic sense system that consists of iron-containing dendrites at six specific bilateral loci. This conclusion, which is supported by a critical analysis of the elemental composition of PB-positive cells in the subepidermis²⁸, has several important implications. First, it requires a re-evaluation of behavioural studies that have purported to impair the function of a magnetite-based receptor in the subepidermis of the upper beak and the conclusions that these studies reached^{9,20}. Second, it necessitates a re-assessment as to whether superparamagnetic magnetite has the necessary physical and magnetic properties to act as a magnetosensor in a living system^{10,11,19}. Third, our work reveals that the sensory cells that are responsible for trigeminally mediated magnetic sensation in birds remain undiscovered. These enigmatic cells may reside in the olfactory epithelium, a sensory structure that has been implicated in magnetoreception in the rainbow trout²⁹.

METHODS SUMMARY

Histological studies. We perfused adult pigeons with 4% phosphate-buffered paraformaldehyde (PFA, pH 7.4), and dissected the tissue with ceramic-coated tools. We embedded the tissue in paraffin and prepared 10- μ m coronal sections. We stained sections for 20 min in 5% potassium hexacyanoferrate with 10% HCl, followed by a series of washes and a 2 min exposure to NFR. For immunohistochemistry we incubated the sections with the primary antibody for 18 h in 0.1% Triton PBS with 2–4% milk (pH 7.4), before detection using standard methods, followed by PB staining.

Ultrastructure studies. Adult pigeons were perfused with 2.5% glutaraldehyde supplemented with 2% PFA in PBS (pH 7.4). Tissue was dissected with ceramic-coated tools, incubated for 1 h in phosphate-buffered 2% osmium (pH 7.4), dehydrated and embedded in epoxy resin. We prepared alternative semi-thin (2 μ m) and ultra-thin (70 nm) sections. Semi-thin sections were stained with PB, and the ultra-thin sections were used for TEM, EFTEM and SAED.

Imaging studies. Adult pigeons were perfused with 4% PFA. Following post-fixation and mounting, MRI imaging was performed on a horizontal bore 9.4 T DirectDrive VNMRs system (Agilent Technologies), and CT on a Nucline Nano SPECT/CT imaging system (Mediso).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.A.K. and C.D.T. conceived and designed the study. M.C.S., C.D.T., M.B., P.P., C.S. and N.E., performed the sectioning, PB staining and counting. D.A.K., C.D.T. and H.C. analysed the resultant data. J.R. and M.L. performed the MRI and CT studies, producing the three-dimensional structure of the pigeon beak. D.A.K. performed the immunohistochemical studies. C.D.T. performed the ultrastructure experiments and J.S. and M.S. did the EFTEM and SAED studies and analysed the data. D.A.K. wrote the paper, and all authors commented on the manuscript.

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METHODS

PB staining. We optimized our PB-staining protocol on our large cohort of pigeons ($n = 172$) that we sourced from seven different lofts. We experimented with a variety of different fixatives, section thickness, and blades before adopting the following protocol. Adult homing pigeons ($n = 12$ Nuremberg cohort, $n = 6$ Vienna cohort) were killed before perfusion with 4% phosphate-buffered paraformaldehyde (PFA, pH 7.4). The tissue was dissected with ceramic-coated tools and post-fixed for 18 h before dehydration in an increasing alcohol series. The tissue was then embedded in paraffin and sectioned coronally (10 μm) with ceramic-coated microtome blades (DuraEdgeHigh Profile, BLM00103P). Four consecutive coronal sections were mounted on positively charged microscope slides (Menzel Superfrost PLUS, Thermo Scientific). For PB staining we incubated every third slide in a freshly prepared solution of 5% potassium hexacyanoferrate (Sigma, P9387) in 10% HCl for 20 min. After three washes in double distilled H_2O , sections were counterstained for 2 min in NFR (Sigma, 60700). Each slide was then scanned (MIRAX Slide Scanner) and PB-positive cells counted manually, using light microscopy where necessary. For anatomical mapping, landmarks were identified (Supplementary Fig. 5), and 500- μm -thick increments determined. To obtain estimated total cell counts the number of PB-positive cells counted within a normalized increment were divided by the number of sections counted within that increment and multiplied by a factor of 50 (see Supplementary Tables 1 and 2). We compared the number of PB-positive cells in males and females by performing a Student's *t*-test. All pigeons were sexed using genetic methods as previously described³⁰, and experiments were performed in accordance with the relevant guidelines and regulations (Magistrat 60, Veterinäramt, MA60-001603/2010/002).

Immunohistochemistry. For staining with neuronal antibodies slides were deparaffinized and washed in PBS (pH 7.4) before incubation with the primary antibody for 18 h in 0.1% Triton PBS with 2% milk (pH 7.4). Primary antibodies were used at the following concentrations: NF (Millipore, MAB1621, 1:2,000), TUBB3 (Covance, MMS-435P, 1:1,000), MAP1B (Santa Cruz, SC-58784, 1:75). Following a series of washes in PBS, slides were incubated for 2 h with a biotinylated secondary antibody (1:500), before visualization with a peroxidase-based Vectastain Elite ABC kit (Vector Labs, PK-4002) and the chromophore DAB (3,5-diaminobenzidine, Dako). For MHCII staining, 10- μm cryosections were prepared, quenched in 2% H_2O_2 in PBS for 30 min, before incubation for 18 h in 0.1% Triton PBS with 4% milk (pH 7.4) with the primary antibody (Santa Cruz, SC-59323, 1:500). This antibody is a mouse monoclonal antibody raised against white blood cells originating from the chicken. To avoid cross reaction with endogenous biotin/avidin, a HRP-conjugated secondary antibody was used (Biorad, 1:500), and staining visualized with DAB. Sections were thoroughly washed with PBS before PB staining and scanning as described above. All cell counting and co-localization studies were performed blinded to the antibody used. The overall percentage of co-localization was determined by calculating the rate of co-localization per bird, and ascertaining the mean.

Ultrastructure studies. Adult pigeons were perfused with 2.5% glutaraldehyde supplemented with 2% PFA in PBS (pH 7.4) (Glut-PFA), before tissue dissection with ceramic-coated tools. Following post-fixation for 48 h, this tissue was washed with PBS, incubated for 1 h in phosphate-buffered 2% osmium (pH 7.4), dehydrated and embedded in epoxy resin. After polymerization, the blocks were trimmed and sectioned, alternatively taking semi-thin (2 μm) and ultra-thin (70 nm) sections. The ultra-thin sections were mounted on formvar-filmed copper slot grids for TEM, whereas the semi-thin sections were etched with 21% sodium ethoxide in ethanol, rehydrated and stained with PB and NFR. Where necessary, PB staining was intensified by incubating the sections in 0.5% DAB³¹. TEM imaging on ultra-thin sections used a 100 kV electron microscope (FEI Morgagni 268D) with a CCD camera (Morada Olympus-SIS). For EFTEM imaging and selected area electron diffraction, the ultra-thin sections were mounted on holey carbon-filmed copper finder grids (Quantifoil, R3.5/1) and analysed on a 200 kV TEM (JEOL, 2100) fitted with a Gatan Imaging Filter (Tridiem) and CCD camera (Orion SC1000). For EFTEM, bright-field images were taken before obtaining elemental maps for iron, which were acquired using the iron M-edge and generated using the conventional three-window method. Two pre-edge (background) images were acquired at energies of 45 and 50 eV, and the post-edge (signal energy) image was acquired by centring the filter's energy-selecting slit at 59 eV with a slit width of 5 eV (~ 10 s acquisition time). Diffraction data was obtained from iron deposits identified by EFTEM and the data calibrated against a polycrystalline gold standard. **MRI.** Animals were killed and perfused with 4% PFA as described above. Following 18 h of post-fixation, heads were mounted in a 70-mm diameter PE tube filled with proton-free perfluoro-polyether fomblin (Solvay Solexis S.p.A.). Imaging was performed on a horizontal bore 9.4 T DirectDrive VNMRs system (Agilent Technologies) using a 72 mm quadrature birdcage volume coil (RAPID Biomedical GmbH). For three-dimensional imaging, a gradient-echo sequence with the following parameters was used: time to echo (TE), 2.8 ms; time to repetition (TR), 280 ms; flip angle, 40°; six averages, field of view, 70 \times 70 \times 35 mm; matrix size, 512 \times 512 \times 256. For higher-resolution images of the beak, PFA-fixed beaks were incubated with 8 mM gadolinium solution (Magnevist, Bayer AG) in PBS for 48 h followed by embedding in agar containing 8 mM gadolinium. Gradient-echo images were acquired using the following parameters: TE, 2.7 ms; TR, 25 ms; flip angle, 45°; five averages; field of view, 28 \times 28 \times 35 mm; matrix size, 560 \times 560 \times 700. Regions of interest were segmented using thresholds with manual adjustments where necessary using Amira visualization software (v.5.2.2, Visage Imaging).

CT imaging. CT imaging was performed on a Nucline Nano SPECT/CT imaging system (Mediso) using the following imaging parameters: 360 projection, pitch 0.5, 55 kVp, 145 μA acquired at 45 μm isotropic reconstructed to 50 μm isotropic.

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