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Testing for the presence of magnetite in the upper-beak skin of homing pigeons

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Abstract

We carried out magnetic and nonmagnetic experiments on fresh, upper-beak skin tissue samples isolated from six pairs of homing pigeons to test whether the tissue contains magnetite particles. Results of (1) room-temperature isothermal remanent magnetization (IRM) acquisition and alternating field (AF) demagnetization, (2) low-temperature demagnetization of saturation IRM acquired at 5 K in a field of 5 tesla (T) ($SIRM_{5\ K}$) after zero-field cooled (ZFC) and field cooled (FC) treatments, and (3) cycling of the saturation IRM acquired at 300 K in a field of 5 T ($SIRM_{300\ K}$) between 5 and 300 K, indicate the presence of magnetite in the measured samples. A significant loss of $SIRM_{5\ K}$ below 20 K suggests the dominance of superparamagnetic (SPM) particles. The SIRM acquisition capacity of the female pigeon is stronger than that of the male pigeon in all four measured pairs, suggesting for the first time that the magnetite concentration is probably sex dependent. Light microscopic observation on the histological sections stained with Prussian Blue detected the presence of some tiny, dotted, dark-blue staining Fe^{3+} aggregates (size 1–4 μm) located directly beneath the subcutis within strands of connective tissue, nearby the rim of the regions full of red nuclei. The results of this study support the idea that homing pigeons may have a magnetite-based receptor, which potentially could be used for sensing the Earth's magnetic field during navigation.

Introduction

A number of previous studies have shown that homing pigeons, *Columba livia*, can make use of the Earth's magnetic field for orientation (Keeton *et al.* 1974; Walcott & Green 1974; Wiltschko & Wiltschko 1995 and references therein). Walcott *et al.* (1979) first detected magnetite in the pigeon head using remanence measurements, electron probe analysis and Curie temperature determination (575 ± 10 °C). The magnetite was thought to be in an innervated piece of tissue between the dura and the skull of the pigeon head. Presti & Pettigrew (1980), however, suggested that magnetite may exist in the tissues between the complexus muscle and the superficial fascia throughout the entire head. Several subsequent electrophysiological studies gave valuable clues in locating the iron-containing structures. Semm *et al.* (1984) and Semm & Damaine (1986) found that the nucleus of the basal optic root which innervates the mechanoreceptors in the skin of the upper beak responded to earth-strength magnetic stimulation. Mora *et al.* (2004)'s recent work also supports the involvement of the ophthalmic branch of the trigeminal nerve in carrying magnetic information to the brain.

Using transmission electron microscopy (TEM), light-microscopy and low-temperature

magnetic measurements, Fe^{3+} concentrations were found in the subcutis of the upper-beak skin of the homing pigeon (*Columba livia*) as aggregates of superparamagnetic magnetite (SPM) (with grain sizes between 1 and 5 nm) (Hanzlik *et al*. 2000; Winklhofer *et al*. 2001; Fleissner *et al*. 2003). The SPM particles formed elongated clusters 1–3 μm in diameter and Winklhofer *et al*. (2001) proposed that such a cluster could represent the core of a magnetic-field receptor. Later, Fleissner *et al*. (2003) conducted an ultrastructural analysis on the subcellular organization of afferent trigeminal terminals in the upper beak, and also proposed a SPM-based magnetoreceptor. Williams & Wild (2001) identified iron deposits in different locations in the pigeon head, the caudal part of the beak, and also some concentrated in cells innervated by ophthalmic nerve fibres, which further suggested that the magnetic sense could be mediated via the trigeminal system.

In this paper, we report experimental results of a series of room- and low-temperature rock magnetic experiments in conjunction with the Prussian Blue (PB) reaction test on fresh, upper-beak skin tissues of homing pigeons, *Columba livia*. Our findings confirm the presence of SPM magnetite in the upper-beak skin and support the idea that the homing pigeon may have a magnetite-based receptor.

Materials and methods

Six pairs of adult homing pigeons,*Columba livia*, were bought from a pigeon market (Bei Sha Tan, Beijing). We bought pairs of pigeons (one male and one female) each time from the same incubation group to enable comparison between the sexes. And we bought all six pairs from the same pigeon breeder. The gender of the pigeons was identified pre-mortem by their physical appearance and standard discriminatory behavior (Wang & An 2004). The upper-beak skin samples were isolated according to the methods of Hanzlik *et al*. (2000) using titanium blades and forceps to avoid iron contamination of the probe. Samples taken from the four pairs were used for magnetic experiments with the other two pairs being used for paraffin sections.

Magnetic measurements were conducted on fresh samples immediately after decapitation at the Paleomagnetism and Geochronology Laboratory (Beijing). Stepwise acquisition of isothermal remanent magnetization (IRM) at room temperature was conducted using a 2G pulse magnetizer in 21 steps up to 1 T. The IRM was subsequently demagnetized using alternating field (AF) demagnetization. Remanence was measured with a SQUID magnetometer (2G Enterprise) (magnetic-moment sensitivity: 10^{-9} emu or in SI units 10^{-12} Am^2) located in a magnetically shielded room (< 300 nT). Due to the magnetic weakness of the pigeon samples, we minimized any magnetic influence of the sample container by using a new sample container with remanence less than 5×10^{-8} emu for each sample. Additionally, we measured and subtracted at each sample measurement step the mean magnetic signal due to the empty sample holder. That is, three empty sample holders were measured and their mean magnetization used as the background to be removed from the sample plus sample holder measurements to leave just the magnetic signal from the sample.

Low temperature (LT, 5–300 K) magnetic measurements are a very useful method for magnetic mineral identification and magnetic granulometry determination. For example, the magnetic behavior of magnetite exhibits both a structural phase transition at $\sim$120 K, called the Verwey transition (T_v), and a magnetic isotropic point at $\sim$130 K (Dunlop & Özdemir 1997). LT measurements were conducted using a Quantum-Design MPMS-XL SQUID magnetometer with a sensitivity of 2×10^{-11} Am^2. The thermal demagnetization of saturation isothermal remanent magnetization (SIRM) acquired in an applied field of 5 T at 5 K ($SIRM_{5\,K}$) after being 'zero-field cooled' (ZFC) and 'field cooled' (FC) treatments in a constant field of 5 T was measured at intervals of 2.5–5 K. The remanence cycling curves ($300 \rightarrow 5 \rightarrow 300$ K) of the SIRM acquired in a field of 5 T at 300 K ($SIRM_{300\,K}$) were measured at intervals of 5 K. Due to the magnetic weakness of the samples, we took sample measurements with automated background subtraction (ABS) (ref. MPMS XL reference manuals-Background Subtraction). The ABS feature facilitates the collection of a sample holder's response (background data) and the subtraction of that data from a combined (sample + sample holder) measurement, thus yielding the magnetic moment of just the sample.

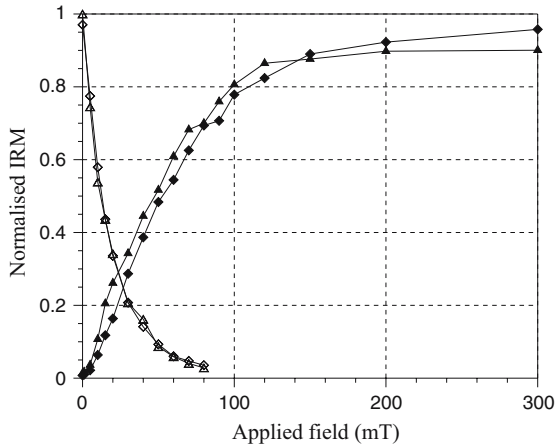

Figure 1. Room-temperature SIRM acquisition (filled symbols) (the maximum applied field is 1 T) and AF demagnetization (open symbols) of pair four samples. Diamonds and triangles stand for female and male pigeon samples, respectively. Remanence from the sample container has been subtracted. Net remanence shown here was normalized by the $SIRM_{1\ T}$. Samples were saturated by a field of 200 mT.

To map areas of iron enrichment, samples from two pigeon pairs were analyzed. The skin samples were embedded in paraffin from which serial horizontal and sagittal sections (10 μm thick) were cut using Leica rotary microtomes (RM2126) (for further preparation details, see Hanzlik *et al.* 2000). The sections were then tested histochemically for concentrations of Fe^{3+} using the PB reaction which is the specific stain test for iron. Any iron-deposits present will appear dark blue in color. Following Bunting (1949) we mixed equal volumes of 2% sodium ferrocyanide and 2% HCl together. After the PB reaction, these sections were counterstained with kernechtrot (Romeis 1968) for histologic recognition of cellular nuclei. The sections were evaluated under an Olympus microscope BX51 (Olympus, Japan) with a DP70 digital camera.

Results

IRM acquisition and demagnetization at room temperature

During IRM acquisition samples reached ~90% saturation with a pulse field of 200 mT (Figure 1). The SIRM values acquired at 1 T range from 24 to 494 $\times$ 10^{-7} emu/g (Table 1). AF demagnetization of SIRM shows a fast decay of initial remanence, with less than 20% of the remanence left after demagnetization by an AF of 30 mT. This suggests that the remanence carriers in the samples are magnetically soft minerals (probably magnetite or maghemite) (Dunlop & Özdemir 1997). Moreover, the mean SIRM of the female sample group (259 $\pm$ 84) $\times$ 10^{-7} emu/g is larger than that of the corresponding male group (124 $\pm$ 50) $\times$ 10^{-7} emu/g (Table 1) though the small sample size did not permit any statistical comparisons.

Table 1. Magnetic properties of the upper-beak skin samples of homing pigeons.

Samples	Sex	$SIRM_{RT}$ (1T)	$SIRM_{5\ K}$ (ZFC_5T)	$SIRM_{5\ K}$ (FC_5T)	δ_{FC}/δ_{ZFC}	Initial $SIRM_{300\ K}$	$SIRM_{300\ K}$ loss
Pair (1)	Male	240	365	424	1.93(0.168/0.087)	100	57.6%
	Female	494	681	795	1.03(0.232/0.226)	120	62.0%
Pair (2)	Male	60	138	191	–	56	51.2%
	Female	141	504	590	1.32(0.330/0.250)	109	65.5%
Pair (3)	Male	24	162	–	–	58	44.3%
	Female	132	185	–	–	60	46.6%
Pair (4)	Male	172	297	293	–	69	58.3%
	Female	270	517	618	1.28(0.345/0.269)	166	65.7%
Male group (mean $\pm$ SE)		124 $\pm$ 50	241 $\pm$ 54	303 $\pm$ 67	–	71 $\pm$ 10	–
Female group (mean $\pm$ SE)		259 $\pm$ 84	472 $\pm$ 104	668 $\pm$ 64	–	114 $\pm$ 22	–

SIRM, saturation isothermal remanent magnetization; ZFC and FC, zero-field cooled and field cooled treatments. δ is the difference between remanences at $M_{80\ K}$ and $M_{150\ K}$ divided by $M_{80\ K}$. Remanence unit, 10^{-7} emu/g. The data for the male and female groups expressed as mean $\pm$ SE. (SE: standard error).

Demagnetization of $SIRM_{5\ K}$ at low-temperature

Samples were first cooled from 300 to 5 K in a 'zero-field' (ZFC) treatment and then an SIRM was acquired at this temperature in a pulse field of 5 T, $SIRM_{5\ K}$. Thermal demagnetization of the $SIRM_{5\ K}$ was measured at intervals of 2.5–5 K during warming from 5 to 300 K in 'zero-field'. The same sample was then cooled from 300 to 5 K in a constant field of 5 T (FC), followed by thermal demagnetization from 5 to 300 K in 'zero-field'. For each sample, $SIRM_{5\ K}$ values after FC treatment are 16–38% larger than that after ZFC treatment. Typical demagnetization curves of $SIRM_{5\ K}$ after ZFC and FC treatments are shown in Figure 2. The $SIRM_{5\ K}$ rapidly decays around the temperature interval 5–20 K. In addition, there is a small loss of remanence in the FC and ZFC curves around 120 K, which indicates the Verwey transition of magnetite (Verwey 1939). The delta ratio was calculated after Moskowitz *et al.* (1993) and yielded values between 1 and 1.3 (except the male 1 sample (1.9)). In both ZFC and FC curves for all samples, we noted a peak around 55 K. This abnormality could be explained by oxygen contamination in the sample chamber of the MPMS.

Interestingly, similar to the room-temperature measurements the female samples have magnetization nearly double that of the corresponding male samples at low-temperature (Table 1). For example, the mean $SIRM_{5\ K}$ values after ZFC treatment for the female and male groups are $(472 \pm 104) \times 10^{-7}$ emu/g and $(241 \pm 54) \times 10^{-7}$ emu/g, respectively. For the $SIRM_{5\ K}$ after FC treatment, the mean values are $(668 \pm 64) \times 10^{-7}$ emu/g and $(303 \pm 67) \times 10^{-7}$ emu/g, respectively.

Cycling of $SIRM_{300\ K}$ (300-5-300 K)

The zero-field cooling–warming cycling curves of $SIRM_{300\ K}$ of sample female 4 are shown in Figure 3. The remanence rapidly decreased between 140 and 120 K, which may be related to transitions of magnetite (Verwey 1939). It then stayed nearly constant down to 30 K before decreasing again down to 5 K to ~60% of the initial remanence value. Upon warming, the remanence showed partial recovery up to 200 K and then decreased up to 300 K. The memory of the $SIRM_{300\ K}$ at room temperature ranges

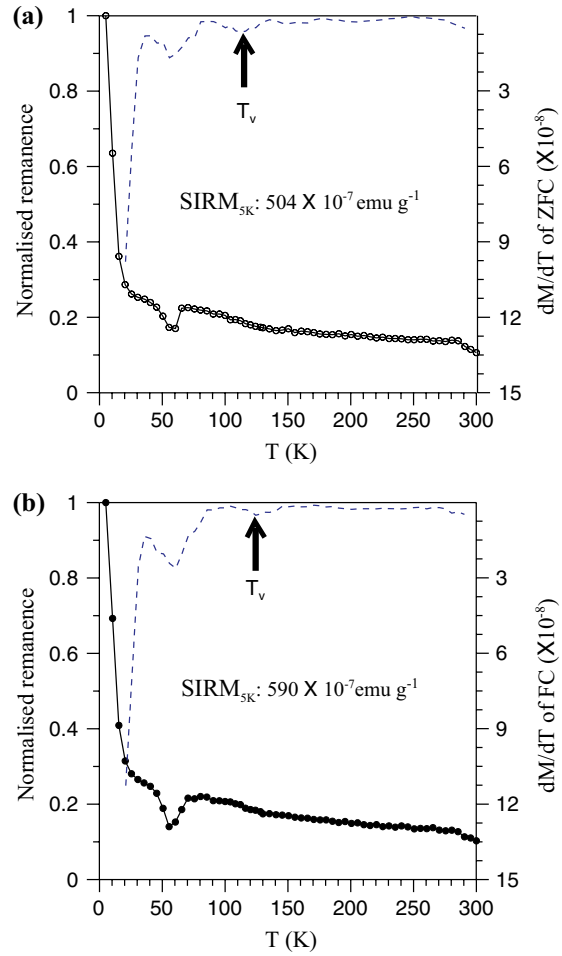

Figure 2. Thermal demagnetization curves of $SIRM_{5\ K}$ (acquired in a field of 5 T at 5 K) and corresponding first derivative curves (dashed lines) of the female two sample. (a) after ZFC treatment (line with open circles) and (b) after FC treatment in 5 T field (line with solid circles). Arrows mark the Verwey transition (T_v).

from 44.3% to 65.7%. Similar to before, it is noted that the female pigeon samples have higher initial $SIRM_{300\ K}$ and remanence loss by cycling compared to the male pigeon samples (Table 1). The mean values of the initial $SIRM_{300\ K}$ for the female and males are $(114 \pm 22) \times 10^{-7}$ emu/g and $(71 \pm 10) \times 10^{-7}$ emu/g, respectively.

Light microscopy

After treating the histological sections with PB, and using highest magnification (100 × oil lens), we found some tiny, dotted dark-blue staining particles localized directly beneath the subcutis

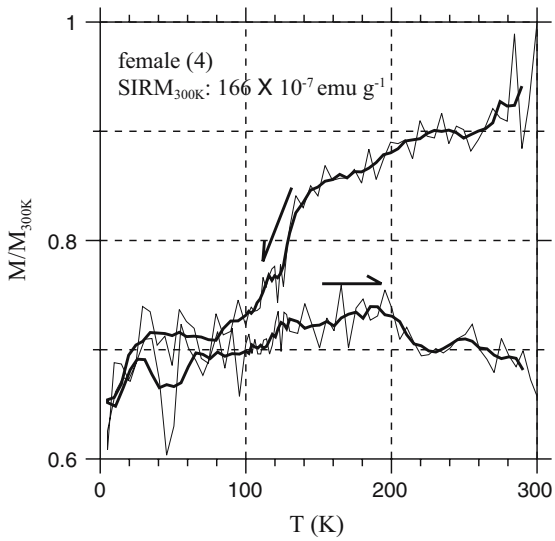

Figure 3. Normalized cooling–warming cycling curves of $SIRM_{300\ k}$, normalized by initial $SIRM_{300\ k}$ (acquired in a field of 5 T at 300 K) of the female four sample. Coarse line is the 5-point running average. Arrows indicate cooling and warming processes.

within strands of connective tissue (Figure 4), nearby the rim of the regions full of red nuclei in a series of five sections. The diameter of the little particles is about 1–4 μm.

Discussion

Our extreme care taken in sample preparations and measurements (e.g., using titanium blades and forceps, and in a specially controlled clean laboratory environment) excludes the possibility of magnetic contamination. Although there is nearly no detectable natural remanent magnetization in fresh samples of the pigeon's upper-beak skin, all samples acquire IRM in ranges of 10^{-6}–10^{-5} emu g^{-1} when exposed to pulse magnetic fields (see Figure 1 and Table 1). Behavior of IRM acquisition (Figure 1) suggests an ordered, blocked ferromagnetic phase, because diamagnetic and paramagnetic phases do not contribute to remanences (Dunlop &

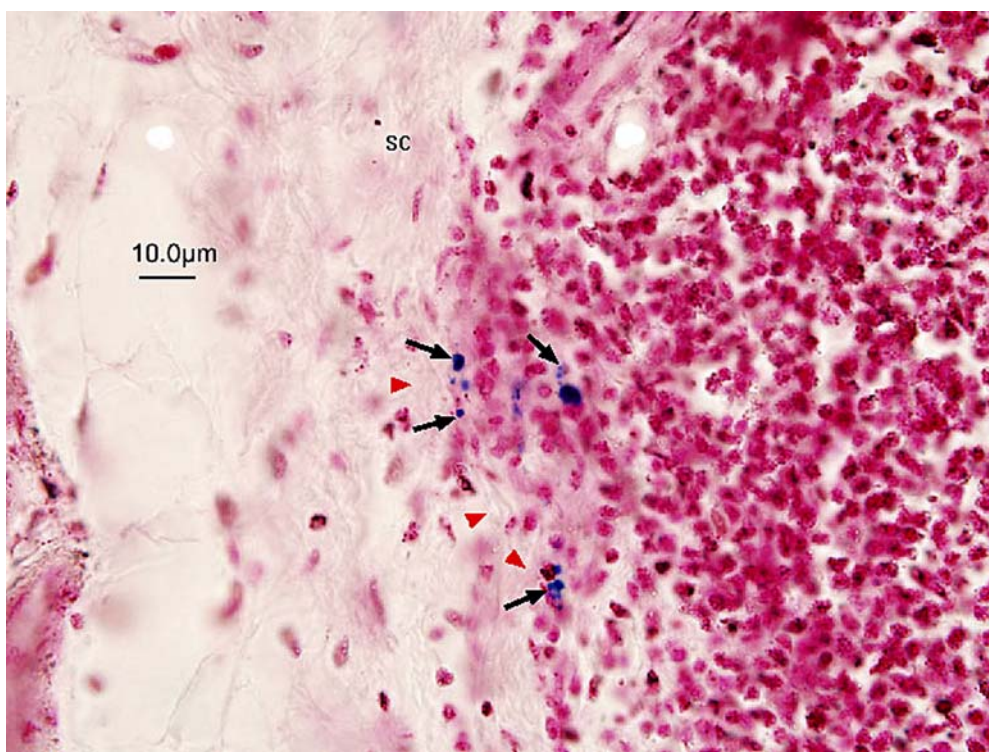

Figure 4. Micrographs of light-microscopy observation on histological sections treated with PB (transmitted light, 100 × oil lens). Tiny dotted dark-blue staining particles (arrows) are localized directly beneath the subcutis (sc) within strands of connective tissue (red arrowheads).

Özdemir 1997). However, Winklhofer *et al*. (2001) noted that the magnetic behavior of pigeon tissue samples after treatment with a pulsed magnetic field cannot be solely attributed to permanent magnetic material such as single domain magnetite, but may reflect the presence of SP material. See the paper of Winklhofer *et al*. (2001) for more detailed discussion.

Our study provides a full set of low-temperature magnetic measurements on the upper-beak skin samples of homing pigeons. Remanence loss around 120 K on the cooling curves of $SIRM_{300\ K}$ and warming curves of $SIRM_{5\ K}$ clearly indicates the presence of magnetite in the measured samples (Figures 2 and 3). A significant rapid decay of $SIRM_{5\ K}$ in the interval of 5–20 K on both ZFC and FC warming curves (Figure 2) suggests the dominance of superparamagnetic (SPM) particles in the samples. Our light microscope observations on paraffin-embeded thin sections showed Fe^{3+} aggregates (size between 1 and 4 μm) in the upper-beak skin samples (Figure 4), but we were not able to increase the microscope resolution sufficiently to determine the particle size and arrangement. Clusters of SPM magnetite particles have been recently identified in the upper-beak skin tissue of *Columba livia* adjacent to nervous material using TEM observations (Hanzlik *et al*. 2000; Winklhofer *et al*. 2001; Fleissner *et al*. 2003). Those SPM particle clusters were of 1–3 μm in diameter, formed by nanocrystal magnetite (1–5 nm). Winklhofer *et al*. (2001) first proposed a qualitative physical model of a postulated magnetoreceptor based on SPM magnetite. Davila *et al*. (2003) recently presented a model where the magnetoreceptor could be based on interacting clusters of SPM magnetite. These authors assumed that approximately 20 SPM clusters formed chain-like aggregates. So far we still do not know if the SPM clusters can carry stable remanence as SD or coarse-grained magnetite can. To prove this, further experimental and theoretical analysis is needed. The low delta ratios (less than 2) calculated from the thermal demagnetization curves of $SIRM_{5\ K}$ in this study do not support a chain arrangement of magnetite crystals as seen in magnetotactic bacteria (Moskowitz *et al*. 1993; Pan *et al*. 2005).

It is very interesting to note for the first time remanence differences between male and female pigeons. Regardless of the temperature at which the remanence was acquired the remanence acquired by the female pigeon samples was larger than that of the corresponding male samples. In this study, one pair of pigeons (one male and one female) was prepared and experiments carried out as an experimental set. Pigeons in each pair were roughly the same age due to each pair from the same incubation group. Distinct differences in SIRM (mainly indicative of magnetite concentration) are clearly seen within an experimental set and also in the group mean values as shown in Table 1. This strongly indicates that the magnetite crystal concentration in the upper-beak skin of homing pigeons is very likely sex-dependent, that is, female homing pigeons have higher magnetite concentration than the male pigeons for our measured samples. We also note distinct magnetic differences between samples from the same gender group (Table 1). Because of unknown exact ages of homing pigeons, potential age factor for the observed discrepancies between and within gender group comparisons can not be clearly described at this stage.

To summarize, in this study light microscopic observation on the histological sections stained with PB and room- and low-temperature magnetic experiments indicate the presence of magnetite (SPM clusters) in the upper-beak skin tissue of homing pigeons (*Columba livia*). This study supports the idea that homing pigeons may have a magnetite-based receptor, which potentially could be used for sensing the Earth's magnetic field during navigation. A comparison between four pairs of homing pigeons (male and female) indicates that the magnetite content in the upper-beak skin may be sex- (and age-) dependent.

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