1 **High levels of maternally transferred mercury disrupt**
2 **magnetic responses of snapping turtle hatchlings (*Chelydra***
3 ***serpentina*)**

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Abstract

The Earth's magnetic field is involved in spatial behaviours ranging from long-distance migration to non-goal directed behaviours, such as spontaneous magnetic alignment (SMA). Mercury is a harmful pollutant most often generated from anthropogenic sources that can bioaccumulate in animal tissue over a lifetime. We compared SMA of hatchling snapping turtles from mothers captured at reference (i.e., low mercury) and mercury contaminated sites. Reference turtles showed radio frequency-dependent SMA along the north-south axis, consistent with previous studies of SMA, while turtles with high levels of maternally inherited mercury failed to show consistent magnetic alignment. In contrast, there was no difference between reference and mercury exposed turtles on standard performance measures. The magnetic field plays an important role in animal orientation behaviour and may also help to integrate spatial information from a variety of sensory modalities. As a consequence, mercury may compromise the performance of turtles in a wide variety of spatial tasks. Future research is needed to determine the threshold for mercury effects on snapping turtles, whether mercury exposure compromises spatial behaviour of adult turtles, and whether mercury has a direct effect on the magnetoreception mechanism(s) that mediate SMA or a more general effect on the nervous system.

Capsule

We show that snapping turtles maternally exposed to high levels of environmental mercury fail to exhibit magnetic alignment compared to reference animals, while performance tests were indistinguishable between groups.

Introduction

Sensitivity to the Earth's magnetic field has been shown in a wide variety of taxa, including turtles¹⁻³. While most studies have focused on the use of magnetic cues for goal-oriented movements, many animals also show spontaneous magnetic alignment (SMA) in which they align their body axis with respect to the Earth's magnetic field (e.g., cattle⁴, foxes⁵, dogs⁶, songbirds⁷, salamanders⁸ and trout⁹). Although the mechanisms underlying SMA, as well as those underlying other magnetic responses, are still under debate, there are two leading hypotheses: 1) a magnetite-based mechanism (MBM), involving single domain or superparamagnetic particles of magnetite¹⁰ and 2) a radical pair mechanism (RPM) in which the Earth's magnetic field influences the spin states of unpaired electrons in specialized

photopigment molecules, potentially altering the response to light¹¹. Both mechanisms can be present in a single animal or, indeed, may be components of a common magnetoreception complex^{8, 12, 13}. In vertebrates other than subterranean mole-rats¹⁴, a MBM is thought to provide the high sensitivity necessary to detect spatial variation in the geomagnetic field used to derive geographic position ('map') information, while the RPM or similar quantum process is thought to be primarily involved in deriving directional ('compass') information^{13, 15, 16}.

Magnetoreception can be involved in many aspects of spatial behaviour: as a compass for local and long-distance movements¹⁷⁻²²; as a source of geographic position information (i.e.), a magnetic map^{23, 24}; as a reference that reduces errors in path integration^{25, 26}; and potentially as a spherical coordinate system that helps to encode the organism's immediate surrounding and to incorporate local landmark arrays into a global map of familiar space^{18, 26}. Consequently, loss of a magnetic sense could impact both long-distance movements (e.g. nesting migrations) and local cue integration.

In a recent study we found that yearling snapping turtles show spontaneous magnetic preferences consistent with SMA in other vertebrates¹⁸. The magnetic response was sensitive to low-level radio frequency (RF) fields at the Larmor frequency, providing compelling evidence for a RPM, or similar quantum process underlying the magnetic compass²⁷. Turtles given their first exposure to the magnetic field in the testing apparatus without RF exhibited SMA when subsequently exposed to the magnetic field without RF, but not when they were exposed to the magnetic field with RF. In contrast, turtles given their first exposure to the magnetic field with RF, only exhibited SMA in the presence of RF, but not without RF. Moreover, the SMA of turtles exposed to RF (i.e., in the 'RF on → RF on' conditions) was oriented towards magnetic south, opposite of that exhibited by turtles in the 'RF off → RF off' conditions, indicating that the turtles were able to derive directional information from the magnetic field in the presence of the RF, but that the pattern of magnetic input was altered in some way. As discussed elsewhere¹⁸ these results are consistent with the earlier proposal that in animals in which a RPM-based magnetic compass is mediated by photoreceptors in the retina, the magnetic field may be perceived as a 3D 'visual' pattern surrounding the animal that appears to be superimposed on the world around it. Moreover, the findings suggest that when turtles find themselves at an unfamiliar (novel) location, they associate the pattern of magnetic input with the novel surroundings. This is analogous to taking a mental 'snap shot' of their surroundings with a perceived magnetic grid superimposed²⁶, similar to retinotopical 'image matching' in insects, which can use magnetic cues as a reference^{28, 29}. Overall, the

emerging picture suggests that magnetic cues are an essential feature of animal spatial perception and behaviour, with a functionality going well beyond ‘classical’ navigational tasks^{5, 21, 25, 26}. Recently it has been shown that anthropogenic pollution in terms of electromagnetic background noise in and near cities are sufficient to disrupt such a magnetic sensor³⁰.

Mercury (Hg) is a highly toxic pollutant released from anthropogenic sources such as waste from chemical plants and emissions from coal burning power plants^{31, 32 33}. In aquatic habitats mercury is methylated into methylmercury (MeHg), which increases its toxicity^{34, 35}. It can bio-accumulate over an animal’s lifetime and be transferred from mother to offspring³⁶. A variety of studies have shown negative effects of mercury exposure on reproduction³⁷⁻⁴⁰ and behaviour^{41, 42} in vertebrates. Investigating whether mercury exposure affects responses to the Earth’s magnetic field is of particular importance not only because of the wide spread effects of mercury toxicity on the vertebrate nervous system⁴³, but also because the effects of mercury may be specific to the magnetoreception mechanism(s) that mediate these responses.

Here we tested whether maternally inherited mercury disrupts the spontaneous magnetic alignment of hatchling snapping turtles. We used turtles hatched in the laboratory from eggs that were collected from mothers with varying levels of mercury to minimize any influence of confounding factors that might differentially affect turtles captured in the field. Siblings of the hatchling turtles used in the magnetic alignment experiment were subjected to commonly used performance tests (righting response and movement speed)^{44, 45}. We show that high levels of mercury eliminate the response of turtles to magnetic cues and discuss this change in response to key spatial stimuli in relation to their ecology and life history.

Materials and Methods

Turtle collection and husbandry

For more detailed information concerning egg collection and husbandry refer to¹⁸. Eggs were collected from females nesting in uncontaminated reference sites along the South and Middle River and Hg-contaminated sites along the South River, VA, USA. In order to quantify maternal and egg Hg concentrations, we collected muscle and blood samples from gravid females and randomly selected three eggs from each clutch (we used 16 contaminated and 18 reference clutches). Muscle samples were collected using a minimally invasive biopsy and were then lyophilized prior to analysis. Blood samples were not prepared further and were analyzed on a wet mass basis. The three eggs from each clutch were lyophilized and homogenized before being pooled as a composite sample. Samples were analyzed for total Hg

at the College of William and Mary, Williamsburg, VA (see ⁴⁶ for additional information regarding Hg analysis). We tested multiple measures of performance of single hatchlings from 12 reference and 12 contaminated clutches. In addition, we tested the SMA responses of 36 reference and 36 contaminated turtles. All females were released after processing and all hatchlings were released at the end of the study at the site of maternal origin.

Performance measures

Locomotor Performance

At 20 days post-hatch, we conditioned hatchlings to the performance protocol before performing the actual performance trial. We placed hatchlings at the start of a 2.3 m sprint track lined with pairs of photocells projecting infrared beams at 10 cm intervals which were interfaced with a laptop computer (Columbus instruments, Columbus, OH; described in full by Holem, et al. ⁴⁷) in an environmental chamber maintained at 25°C. We stimulated hatchlings with a pair of blunt forceps at the base of their tail to elicit a forward locomotor response. Once the hatchling ceased moving, we waited 10 seconds before administering an additional stimulus and repeated this process as needed until the hatchling covered a distance of one meter. After conditioning, individuals were returned to their respective housing containers.

We conducted the experimental performance trials once hatchlings reached 21 or 22 days post-hatch using the same methods described above for conditioning. We recorded the number of stimuli administered by the investigator using a digital voice recorder. At the completion of the 1 m locomotor performance trial, we measured mass (g), carapace length and width (mm), plastron length (mm), shell depth (mm), and tail length (mm) for each hatchling. We characterized locomotor performance as the maximum velocity over a 10 cm distance.

Righting Response

At 21 or 22 days post-hatch, we placed each hatchling in an individually labeled 591-ml Ziploc® container filled to a depth of 2 cm dry, coarse sand. Containers were labelled with the corresponding hatchling's identity, and arranged in a 2x3 grid that made it possible to test six individuals simultaneously. A digital video camera was suspended above the arena grid to record experimental trials. Individual hatchlings were randomly placed into one of the six containers, and allowed to acclimate for two minutes. Each individual was then turned over

onto its carapace. The observer then stepped behind a raised blind and monitored the trial using an external viewing screen on the digital video camera. Once all hatchlings had righted or 60 minutes passed, the trial was considered complete and each turtle was returned to its respective housing container. The experimental subjects were given two minutes of rest before repeating the trial twice for each hatchling, resulting in three righting response trials per individual.

Three components of turtle righting performance were characterized: 1) time at first righting attempt, 2) mechanical righting response, defined as the active time spent during a successful right, and 3) time to right, classified as the total time between initial inversion and successful righting. Righting response videos were viewed using Adobe® Encore CS5 software (Adobe Systems Incorporated, CA, USA).

Magnetic alignment testing procedure

For detailed description of the procedures used to characterize magnetic alignment responses see ¹⁸. Turtles were tested for magnetic alignment in individual chambers; each consisted of a vertically aligned PVC tube that formed the walls of the enclosure, set inside a Pyrex bowl containing about 1 cm of dechlorinated water, and covered with a frosted glass diffuser (see previously published methods ¹⁸). On each day of testing, twelve turtles were tested simultaneously, six were from clutches with low levels of mercury contamination (reference animals), and six were from clutches with high levels of mercury contamination. Each animal experienced all four magnetic field alignments, magnetic north = topographic north, magnetic north = topographic east, magnetic north = topographic south and magnetic north = topographic west (in one trial in each alignment of the magnetic field the turtles were not exposed to the RF stimulus, while in a second trial in each magnetic field alignment they were exposed to the RF stimulus) and the vertical field in a pseudo-random order. This resulted in nine different conditions, to each of which the turtles were exposed for at least one hour. Half of the tested turtles were pre-exposed to RF when first introduced to the testing environment ('RF on →'), the other half were not ('RF off →'). The directional responses from the four magnetic field alignments in each RF exposure combination were pooled for each condition and turtle, resulting in five conditions: 'RF off → RF off', 'RF on → RF on', 'RF off → RF on', 'RF on → RF off' and the vertical field.

Magnetic fields were generated using a pair of horizontally aligned, double-wrapped Rubens coils wrapped on the same frame ^{48, 49}, which produced four fields with mean field strengths of $51.24 \pm 0.06 \mu\text{T}$ (the vertical field had a strength of $46.78 \mu\text{T}$), aligned with

magnetic north at topographic (= geographic) north, east, south and west. Radio frequencies were produced with a signal generator (Agilent, model 33250a), amplifier (Amplifier Research Associates, model 10A250), and a horizontal loop antenna. In the ‘RF on’ condition we generated frequencies at the Larmor Frequency (1.430 MHz) at intensities that ranged from about 30 nT to 52 nT depending on the location of the testing chamber inside the RF coil.

Trials were recorded by a video camera and the last 40 minutes from each experimental condition were used for further analysis; 36 frames (obtained at a frame rate of 0.9 per minute) out of each recording were analysed. We defined the alignment direction in each frame as the straight line between the base of the tail to the turtle’s neck.

We also determined the posture of turtles for each frame; directional data from postures categorized as “probably moving – direction not measureable” and “crawling up against the wall” were excluded from further analysis. Over the whole course of the experiment 3.5 % of the measurements were discarded (824 out of 23328) due to these reasons. All measurements were collected with the experimenter blind to the turtle’s real identity, order of testing, level of mercury exposure, and stimulus condition (i.e., magnetic field alignment, and presence or absence of RF field). JBP and MSP assigned arbitrary numbers to mercury and reference turtles as well as trials. The identity of the trials and turtles were only revealed after alignment measurements were completed by LL.

Statistical Analysis

We used SAS 9.2 (SAS Institute, Inc, Cary, NC, USA) for all statistical analyses regarding the performance measures. Significance was assessed at $\alpha \leq 0.05$. When appropriate, we $\log_{10}$ -transformed performance variables to improve normality and homoscedasticity. Initial models included hatchling body size (carapace length) as a covariate, but this term was later removed from all final models due to non-significance.

We compared Hg concentrations between females and their eggs collected from reference sites and contaminated sites using three one-way analysis of variance models (ANOVA; SAS PROC GLM) with site as the main effect and tissue type (i.e., muscle, blood, egg) as the response variable.

We tested for the effects of maternal site of origin on hatchling performance. In order to determine the influence of Hg on locomotor performance, we used an analysis of variance (ANOVA; PROC GLM) with female collection site (reference or contaminated) as the main effect. To understand/investigate the influence of site of origin on hatchling righting response

measures, we used a multivariate analysis of variance (MANOVA; Pillai's Trace; SAS PROC GLM). The model included time at first righting attempt, mechanical righting response, and time to right as response variables and female collection site as the main effect.

To assess significant magnetic alignment we first calculated the vector-sum for each animal for each condition described earlier ('RF off → RF off', 'RF on → RF on', 'RF off → RF on', 'RF on → RF off' and the vertical field). The vector-sum was calculated by simple vector addition from the individual alignments (one alignment direction for each frame) in each experimental condition (see above and Landler, Painter, Youmans, Hopkins and Phillips¹⁸). For each RF treatment results of the four magnetic field directions were combined, either calculated in relation to the changing magnetic north, or the unaltered topographic north. Using this procedure a magnetic response can be separated from a magnetic response⁵⁰. The resulting vector distributions were tested for departure from a random distribution using the Moore's modified Rayleigh-test⁵¹, paired observations were tested for a difference in the distribution of responses in the two experimental treatments (i.e., RF off and RF on) using the Moore's paired sample test. Independent observations (e.g. comparison between reference and mercury animals) were tested using the Mardia's two-sample comparison test⁵². For all circular statistics Oriana 4 was used. See¹⁸ for additional details of the statistical analysis.

Results

Total Hg concentrations in gravid female tissues collected from the reference and contaminated sites averaged 0.14 ± 0.01 ppm and 15.61 ± 2.47 ppm (dry mass) for muscle and 0.02 ± 0.01 ppm and 2.26 ± 0.23 ppm (wet mass) for blood, respectively. Egg Hg averaged 0.04 ± 0.01 ppm (dry mass) for reference clutches and 3.26 ± 0.25 ppm for contaminated clutches. In all cases, tissue concentrations were significantly higher in turtles from the contaminated site compared to those from the reference site ($n=12$, female muscle: $F_{1,23}=322.9$, $p < 0.001$; female blood: $F_{1,23}=293.9$, $p < 0.001$; egg: $F_{1,23}=593.4$, $p < 0.001$). However, collection site did not influence locomotor performance ($n=12$, $F_{1,23}=0.64$, $p=0.43$, figure 1) or righting response ($n=12$, $F_{3,20}=0.95$, $p=0.44$, figure 1) of turtle hatchlings.

In an earlier paper¹⁸, we showed that turtles from the reference site aligned their body axes in a consistent direction relative to the magnetic field when tested in the same RF condition they experienced when first introduced to the testing apparatus (i.e. in the 'RF on → RF on', and in the 'RF off → RF off' conditions; fig 2), while turtles from the reference sites were disoriented when tested in an RF condition that differed from that initially experienced

in the testing apparatus (i.e. ‘RF off $\rightarrow$ RF on’ and ‘RF on $\rightarrow$ RF off’; data not shown, but see¹⁸). In contrast, mercury turtles did not show consistent magnetic alignment in any of the RF conditions; figure 2 shows the distribution of responses exhibited by reference and mercury exposed turtles in the ‘RF on $\rightarrow$ RF on’ and ‘RF off $\rightarrow$ RF off’ conditions and see¹⁸. The difference in the responses from reference and mercury turtles in the ‘RF off $\rightarrow$ RF off’ condition was significant ($n=18$, $U = 0.276$, $p < 0.01$, Mardia's two sample test, figure 2) and approached significance in the ‘RF on $\rightarrow$ RF on’ conditions ($n=18$, $U = 0.177$, $p < 0.1$, Mardia's two sample test, figure 2). In the two conditions in which turtles were exposed to a RF condition that differed from that experienced when they were first introduced to the testing apparatus (i.e. in the ‘RF off $\rightarrow$ RF on’, and ‘RF on $\rightarrow$ RF off’ conditions), both reference and mercury exposed turtles were disoriented, and their responses were not significantly different.

The distribution of alignments of reference turtles relative to topographic north (i.e. ignoring the alignment of the magnetic field) were indistinguishable from random in all four conditions while animals collected from the mercury site were significantly aligned relative to topographic north in one of four testing conditions ($n=18$, ‘RF off $\rightarrow$ RF on’ condition; $R^* = 1.308$, $p < 0.01$, Moore’s modified Rayleigh test, figure S1; and see S2 and S3).

Discussion

Yearling turtles from the reference sites exhibited RF-dependent SMA¹⁸, but turtles from the mercury-contaminated sites failed to exhibit consistent alignment relative to the Earth’s magnetic field in any of the conditions tested. As discussed in the earlier paper, in reference turtles, RF exposure appeared to alter, rather than eliminate, the directional information obtained from the magnetic field¹⁸; turtles first exposed to the magnetic field in the testing apparatus with ‘RF off’, subsequently exhibited SMA in the four magnetic field alignments with no RF, and not in the four magnetic field alignments with RF present. In contrast, turtles first exposed to the magnetic field with RF, only exhibited SMA in the four magnetic field alignments with RF, and were indistinguishable from random in the four magnetic field alignments without RF. Moreover, while reference turtles tested in the ‘RF off $\rightarrow$ RF off’ condition exhibited northward SMA, reference turtles tested in the ‘RF on $\rightarrow$ RF on’ condition exhibited southward SMA, providing further evidence that exposure to the RF field altered the directional information obtained from the magnetic field, rather than eliminating this input altogether. Evidence that turtles associate the (RF-dependent) pattern of magnetic input with a novel location is consistent with the proposed roles of the magnetic

field in: (a) helping to organize spatial information when an animal finds itself at a new location (i.e., typically it would be surrounded by unfamiliar landmarks), and (b) providing a global reference system that can play an important role in assembling such local landmark arrays into a global map of familiar space²⁶.

The biological importance of SMA is not very well understood. It might be involved in anchoring the visual surrounding on a fixed magnetic direction, and cue integration²⁶. However, given the importance of the magnetic field in long distance orientation in all of the major vertebrate groups, including turtles (e.g., providing primary sources of both map and compass information, as well as serving as a calibration reference for other compass systems⁵³), the failure of mercury exposed turtles in the present experiments to respond to the magnetic field in any of the testing conditions (RF on or RF off) suggests mercury exposure could have deleterious effects on a wide variety of spatial movements (migration, dispersal, foraging, mate-finding, etc.). Moreover, the finding that mercury exposure prevented turtles from associating the pattern of magnetic input (i.e., either with RF on or with RF off) with novel surroundings suggests that the impact of mercury exposure on spatial behavior and perception could be more general than previously realized, compromising turtles' use of spatial information at multiple spatial scales and at all life history stages.

The absence of SMA (exhibited by reference animals) in mercury exposed turtles (Fig 2), in contrast to the absence of any significant differences between reference and mercury-contaminated turtles in righting response and movement speed raises the interesting possibility that at least some of the effects of mercury could be specific to the magnetoreception system(s).

Both magnetite-based (MBM) and radical pair mechanisms (RPM), as well as a hybrid mechanism involving both of these magnetically sensitive processes, could be directly impacted by mercury exposure. Magnetite has a strong affinity for mercury, as shown by its utility in cleaning up mercury spills⁵⁴, making it possible, and indeed likely, that mercury could interfere with the functioning of a MBM. The same is true for a RPM, which appears likely to play at least some role in the turtles SMA¹⁸; and see earlier discussion. The putative receptive molecule for a RPM is cryptochrome⁵⁵⁻⁵⁸, a molecule best known for its role in circadian rhythms⁵⁹. The involvement of cryptochrome in the magnetic compass is consistent with most behavioral and histological evidence⁵⁵. Recently, it has been shown that a Zinc ion serves as a cofactor in cryptochrome photochemical reactions⁶⁰. Zinc is also involved in the heterodimer formation of cryptochrome and period proteins, which plays a central role in the circadian clock and its photo-entrainment to the light:dark cycle⁶⁰. Displacement of the Zinc

cofactor due to competitive binding with mercury⁶¹ could disrupt the interactions of cryptochrome with a signalling partner. Finally, if SMA of turtles involves both magnetoreceptor systems (RPM and MBM¹³), mercury could interfere by binding to one or both of these components^{62, 63}.

While investigating whether effects of mercury exposure are specific to the sensory mechanism(s) responsible for detection of the geomagnetic field will be an extremely interesting avenue for future research, mercury exposure also has other physiological effects that could influence the responses of turtles to magnetic cues. For example, mercury can negatively affect vision^{64, 65}, as well as learning and memory^{42, 66}, and therefore could disrupt an association between the pattern of sensory input produced by the magnetic field and the visual surrounding. Moreover, our study can exclude that the turtles have even more general defects, which we did not detect in the motor-related performance tests. Thus, future work is needed to disentangle the interactions and relative importance of different effects of Hg that may influence the responses of turtles to magnetic cues.

Mercury levels at the contaminated sites in this study were very high in relation to other examples of mercury contamination [63]. Further research on the effects of lower concentrations of mercury is needed in order to determine the threshold for impacts on wildlife populations. Likewise, because mercury exposure in the present study was entirely of maternal origin, it remains to be determined if dietary exposure to Hg could produce similar effects. Additional studies are also needed to determine if effects of mercury on magnetic spatial behaviour are present at other life history stages. Such questions could be explored by testing, for instance, homing performance of adult snapping turtles displaced from reference vs. mercury contaminated sites.

Recent research suggests that magnetic input may be used by animals not only for long-distance movements and migration, but also to structure spatial perception of the animal's immediate surroundings^{19, 26, 67, 68}. Therefore, the geomagnetic field's role in spatial behaviour and cognition may be more general than previously recognized. Consequently, any environmental contaminant that interferes with the detection or use of this vital sensory input is likely to affect all aspects of the turtle's spatial abilities.

Competing interests

We have no competing interest.

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Notes

The authors declare no competing interest.

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Figure 1: Comparison of hatchling righting response components (A,B,C) and locomotor performance (D) of individuals hatched from females collected from either reference or Hg contaminated sites along the Middle and South Rivers (Waynesboro, VA, USA). Bars represent means $\pm$ 1 S.E.

Figure 2: Comparison between reference animals and mercury contaminated yearling snapping turtle magnetic alignment. Each line (with dotted end) represents the orientation vector of one individual; its length represents the strength of orientation (numbers on dashed concentric lines correspond to vector length in a unit circle). The black arrows represent the second order mean vector of the distribution. The left panel shows the circular distributions of reference animals, the right panel animals affected by maternally transferred mercury. Black lines connected to dots represent responses of turtles in the absence of RF, red lines represent responses in the presence of RF. Red outer circle (labeled 'RF on') represents trials with RF acclimation, black outer circle (labeled 'RF off') represents trials with no RF acclimation. Moore's modified Rayleigh test was used to test each distribution for non-

572 random unimodal alignment. Data were tested for significant differences using the Mardia's
573 two-sample test.

574

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576

577

Figure 1

Figure 2

Reference turtles show
radio frequency dependent
magnetic alignment

$p < 0.01$

A horizontal line with arrows at both ends, pointing left and right, indicating a comparison between the two groups.

Mercury exposed turtles show
no magnetic alignment