Spontaneous Directional Preferences in Taxonomically and Ecologically
Distinct Organisms: Examining Cues and Underlying Mechanisms

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Spontaneous Directional Preferences in Taxonomically and Ecologically Distinct Organisms: Examining Cues and Underlying Mechanisms

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Abstract

The focus of this research was the spontaneous magnetic alignment responses of animals. We show that snapping turtles (*Chelydra serpentina*) and crayfish (*Cambarus sciotensis*) spontaneously align their body axes relative to the magnetic field. In snapping turtles, this response is sensitive to low-level radio frequency fields, consistent with a mechanism involving a light-dependent radical pair mechanism. Findings from the turtle experiments also suggest that the Earth’s magnetic field plays an important role in encoding spatial information in novel surroundings, and may help to organize multiple locales into a ‘mental map’ of familiar space. Given the importance of magnetic input in many aspects of spatial behavior, another important finding was that magnetic alignment of yearling turtles was disrupted by high levels of maternally transferred mercury, an industrial waste product found at high levels in some fresh water ecosystems. In crayfish, we investigated the effects of ectosymbionts (Annelida: Branchiobdellida) on magnetic alignment responses. Interestingly, the response of crayfish to magnetic cues parallels the complex symbiotic interaction between crayfish and their ectosymbiotic worms, which changes from mutualistic to parasitic with increasing worm density. Our working hypothesis was that these changes in spatial behavior may increase or decrease contact to other crayfish, and therefore increase or decrease transmission rates. Next, to address the ontogeny of the SMA, we attempted to replicate an earlier study showing a possible magnetic alignment response in chicken.
embryos. Although chicken embryos did show non-random alignment, we were not able to find a magnetic effect. Alignment is also an important feature of animal constructions and is very likely to have fitness consequences, which we explored in woodpecker cavity alignments in a meta-analysis of available global data. The latitudinal and continental pattern in 23 species of woodpeckers suggests that an alignment response can have the proximate function to regulate microclimate in the cavity and therefore, presumably, optimize incubation temperatures and increase hatching success. Overall, the presented findings show how experimental and observational studies of spontaneous alignment behavior can provide insight into the ecology and sensory biology of a wide range of animals.
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1. Introduction: Possible role of spontaneous magnetic responses in animal spatial behavior

Lukas Landler

For many decades ethologists have been fascinated by the ability of animals to navigate over long-distances and by the variety of cues used for such behaviors [1-3]. Traditionally, migratory birds have been the center of attention, because of innate seasonal migrations [4], many of which cover impressive distances, and their robust behavioral responses in laboratory studies [5]. Animals can use a variety of cues for spatial orientation, including the sun [6], polarized light [7], olfactory cues [8], auditory cues [9] and the Earth’s magnetic field [10]. While the sensory mechanisms are well characterized for many of the cues involved in spatial behaviors, some cues remain enigmatic. For example, early work on European robins and red-spotted newts has provided unambiguous behavioral evidence for the role of magnetic cues in migratory and homing behavior [11,12], however the sensory receptors and biophysical mechanisms mediating magnetoreception have yet to be identified. Interestingly, work from migratory birds and newts suggest that magnetic cues provide both directional (compass) information and positional (map) information [13-15]. While some researchers use the innate migratory behaviors of birds and turtles to study such behaviors [11,16], others have used conditioned responses, in which animals learn to orient relative to a particular trained direction [17-20]. Recently however, there has been renewed interest in
another suite of magnetic response, spontaneous magnetic alignment (SMA) behavior, in which an animal aligns its head direction or body axis with respect to the local magnetic field [21]. Although SMA was first described in the common European cockchafer (*Melolontha melolontha*) over 50 years ago by Schneider [22], it was only recently that researchers began to study the possible functional significance and underlying mechanisms of these behaviors [23-26]. Studies of SMA received much attention in public media mainly due to the apparent ‘curiosity’ of such behaviors (for instance one research group received an Ig Nobel Prize for their research on magnetically aligning dogs [27]). Scientifically however, these behaviors might represent a very important facet of spatial behaviors, and could possible provide key insights to the underlying structure and organization of spatial ecology.

To date there are two main hypotheses about mechanisms of magnetoreception in terrestrial and freshwater animals [28,29]. 1) A magnetite based mechanism (MBM) and 2) a light dependent radical pair mechanism (RPM). The MBM is believed to be based on biogenic single domain or superparamagnetic magnetite particles [28,30,31]. Biological magnets, i.e. chains of magnetite particles, could open ion-channels through cytoskeletal filament connections [28]. The most powerful diagnostic test for MBM are pulse re-magnetization experiments, in which the polarity of the magnetite particles is changed [32]. Other properties consistent with a MBM include non-light dependency, unaffected by radio-frequency exposure (see below), polarity - not inclination - sensitive (see RPM). Beside evidence from a number of animals showing a functional magnetic response under total darkness and long wavelength light (>500 nm) [24,33-37], evidence using pulse re-magnetization only exist from birds, honey-bees and turtles [15,32,38-41]. In mole-rats, in addition to a functional magnetic compass under total darkness, the magnetic response was also shown to be unaffected by radio-frequency exposure [42].
Fleissner et al. [43] reported finding particles of magnetite in the upper beak of birds. In accordance with Fleissner et al. [43] findings, anesthetization of the trigeminal nerve innervating the upper beak was found to disrupt the magnetic map-sense [44], providing support for the presence of a MBM magnetoreceptor in the upper beak of birds. However, more recent findings challenged Fleissner et al. [43] showing that the ferromagnetic structures in the upper beak are most likely macrophages involved in iron homeostasis [45]. Similarly, structures in the inner ear of birds and in the olfactory lamellae of rainbow trout, earlier proposed to constitute magnetite receptors [46-48], may be indicative of iron contamination from sample preparations rather than magnetoreceptors [49]. Therefore, the location of a MBM magnetoreceptor remains in question.

In contrast to MBM, the location of the putative RPM receptor is better understood. First evidence for a light dependent RPM compass came from amphibians [50] where it was also shown that the pineal organ is involved in magnetoreception [51]. However, in birds, the potential receptor molecule, cryptochrome, known for its role in the circadian clock [52], seems to be associated with UV-cones on the retina [53]. The RPM is based on a photo-induced electron transfer reaction that results in the formation of a radical pair [54-56]. If the spin state of the two unpaired electrons is antiparallel, the radical pair is in a “singlet state” and if they are parallel it is in a “triplet state”. The precession frequency of an unpaired electron is influenced by the local magnetic field, which is the result of the Earth’s magnetic field and any magnetic moments of magnetic nuclei in the molecule [55]. In the case of weak hyperfine interactions the precession of the electron spin can be influenced by the Earth’s magnetic field (the Zeeman effect), which leads to changes in the interconversion between singlet and triplet states. The triplet state (= signaling state) is expected to yield a product, which increases or deceases the sensitivity of photoreceptors based on their alignment.
relative to the Earth’s magnetic field. Increasing the triplet character of the radical pair prevents back transfer and also increases the signaling state. The RPM requires relatively long-lived radical pair formations and spin correlation (i.e. interaction between the two electrons) of the radicals involved. Because receptor molecules and photoreceptors on the retina are arranged on a half sphere, the effect of the magnetic field on individual photoreceptors could vary the dependence of the response to light on the alignment in relation to the Earth’s magnetic field. This could result in a three-dimensional pattern that appears to be superimposed on the visual surrounding of animals [54,57,58]. Importantly, this magnetic ‘visual’ pattern would appear to move with the animal but would remain fixed in its alignment relative to the magnetic field. Therefore this pattern is well suited to be used as a
global reference, i.e. pointing towards a consistent directional heading, independent of location [59]. Unlike the MBM, the RPM based compass is sensitive to the axis, not the polarity of the magnetic field; a magnetic compass based on this mechanism cannot distinguish between the magnetic north and south poles [51,60]. A second property that distinguishes the RPM is sensitivity to low-level RF fields in the range of 0.3 to 30 MHz which should interfere with the interconversion between the singlet and triplet states, if the frequency is close to the precession frequency of the electron spin dipole [61]. RF at such frequencies is in resonance with the hyperfine and Zeeman interactions and therefore modifying the spin dynamics of the radical pair [55].

Effects of RF have been studied in a variety of animals, and at least birds have been shown to be affected by RF fields [56,62]. These effects cannot be explained by the alternative, MBM, hypothesis because electromagnetic fields many orders of magnitude higher would be needed to affect the alignment of magnetite particles [61]. It is important to note that the two mechanisms are not mutually exclusive. Evidence from birds and newts
suggests that both mechanisms are present in the same animals, however, having different functions [44]. In animals that possess both mechanisms the RPM provides directional compass information [63], while the magnetite receptor provides information about magnetic intensity (or inclination), which is needed for a map-sense (i.e. the ability of an animal to position themselves on an internal representation of a geographic map) [41,64]. However, subterranean rodents possess only a magnetite based mechanism, which does act as a compass sense [42]. The question that arises from this is: why would animals have two parallel magnetoreception mechanisms, if one of the two (the MBM) could fulfill both functions? While still speculative, it has been argued that this arises from additional functions that the RPM can provide, by the possible advantages of a ‘visual’ 3D pattern in spatial learning and memory formation [59].

   Beside the importance for long distance movements, evidence from trained magnetic assays suggests that the Earth’s magnetic field is involved in a rapid encoding of novel surroundings. For instance, honeybees use the Earth’s magnetic field in order to align themselves in a fixed direction and presumably store a retinotopical mental image of the surrounding when they first encounter a novel environment [65]. In subsequent trials, honeybees retain a preferred alignment, however, they no longer rely on magnetic cues to guide these responses suggesting that magnetic cues may help initial encoding in novel environments [65]. The Earth’s magnetic field therefore, might provide a fixed reference from which to encode other types of cues (e.g. visual cues used for development of a retinotopic map) [66]. Another example comes from mice trained in a modified Morris water maze, in which they are given only two ~30 s training trials to learn the magnetic direction of a submerged platform [18]. These findings are particularly striking, as water maze tasks require days of training to develop reliable spatial responses [67-69]. To explain such
findings Phillips et al. [59] developed a hypothesis which combines the RPM model with the retinotopical image matching; in the proposed hypothesis animals store a mental image analogous to a ‘snapshot’ when first introduced to a novel environment. Because the RPM is thought to produce a visual pattern superimposed on the surrounding, the snapshot forms a compound image consistent with the image of the magnetic input superimposed. Similar to the retinotopic image hypothesis, animals could use the stored snapshot when re-encountering the same environment. Although not required, a more complicated pattern, which some recent evidence points towards [59,62], could facilitate such a process; certain parts of the pattern could be associated with visual landmarks [59]. Increasing familiarity with the environment may reduce the salience of the magnetic field cues and increase attention to stable visual cues (i.e. transfer of magnetic information generated by radical pair mechanism to visual landmarks). Given the existing evidence and current hypotheses surrounding magnetoreception/magnetotactic behavior, it is likely that SMA is more than an arbitrary behavior without any adaptive significance, but rather a behavior of high importance to animals, used to encode the visual surrounding.

While knowledge about the integration of magnetic cues on a neuronal level is poorly understood, current findings and ideas about the spatial representations in animals’ brains share some properties with the ‘snapshot’ hypothesis (some of this is reviewed in Phillips et al. [59]). For instance, hippocampal places cells show specific firing patterns for remembered specific locales, however, when placed into new testing environment (or significantly changed testing environments) place cells ‘spontaneously’ remap a new place pattern, which then becomes fixed for this particular testing environment [70]. This is consistent with the view that multiple locales can be stored in the brain using local maps of the respective immediate surroundings. Furthermore, place cells (and also other neuronal cells for spatial
representation in the hippocampus and subiculum) expand their firing fields when the testing environment is enlarged [71,72]. This would be expected when matching up a stored ‘snapshot’ with a presented environment [65,73]. A second type of spatial cells, head direction cells (HDC), seem to provide the neuronal equivalent of a compass for animals [74]. HDC seem to integrate vestibular input with visual information, however HDC properties may not be explained by those two sensory inputs alone. Further research considering magnetic cues as possible factors in spatial memory consolidation might prove a very fruitful endeavor (Phillips Lab, research in progress).

Free-living animals face a variety of challenges from both, natural occurring (e.g. larger scales) and anthropogenic (environmental toxicants) sources. While Chapter 2 explores the magnetoreception mechanism underlying SMA and the potential function in encoding of novel environments, Chapter 3 investigates the effect of environmental toxicants on this response; Chapter 4 provides evidence that ectosymbiotic worms change the magnetic response of their hosts (crayfish), using similar methods as in the previous chapters. Chapter 5 shows the results of a failed attempt to replicate a previous study on magnetic alignment of chicken embryos; indicating that rigorous magnetic field manipulations are needed to distinguish between a response to magnetic field versus other non-magnetic factors. Chapter 6 investigates the potential proximate factors of alignment responses in a meta-analysis of woodpecker cavity alignments on a global scale; suggesting that an alignment response is likely to be related to thermoregulation and thereby have fitness consequences for the animals.
Evidence from those five chapters taken together suggest that alignment responses can be a very useful tool to provide insight into spatial cognition and behavioral ecology, but also effects of toxicants and species interactions on animal spatial behavior.

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2. Spontaneous magnetic alignment by yearling snapping turtles: rapid association of radio frequency dependent pattern of magnetic input with novel surroundings

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Abstract

We investigated spontaneous magnetic alignment (SMA) by juvenile snapping turtles using exposure to low-level radio frequency (RF) fields at the Larmor frequency to help characterize the underlying sensory mechanism. Turtles, when first introduced to the testing environment without the presence of RF aligned consistently towards magnetic north when subsequent magnetic testing conditions were also free of RF (‘RF off → RF off’), but were disoriented when subsequently exposed to RF (‘RF off → RF on’). In contrast, animals initially introduced to the testing environment with RF present were disoriented when tested
without RF (‘RF on → RF off’), but aligned towards magnetic south when tested with RF ('RF on → RF on'). Sensitivity of the SMA response of yearling turtles to RF is consistent with the involvement of a radical pair mechanism. Furthermore, the effect of RF appears to result from a change in the pattern of magnetic input, rather than elimination of magnetic input altogether as proposed to explain similar effects in other systems/organisms. The findings show that turtles first exposed to a novel environment form a lasting association between the pattern of magnetic input and their surroundings. However, under natural conditions turtles would never experience a change in the pattern of magnetic input. Therefore, if turtles form a similar association of magnetic cues with the surroundings each time they encounter unfamiliar habitat, as seems likely, the same pattern of magnetic input would be associated with multiple sites/localities. This would be expected from a sensory input that functions as a global reference frame, helping to place multiple locales (i.e., multiple local landmark arrays) into register to form a global map of familiar space.
**Introduction**

Turtles are among the wide variety of animals known to be sensitive to the Earth’s magnetic field [1-4]. However, the nature of the underlying magnetoreception mechanism has yet to be determined [5-7]. In terrestrial animals, there is evidence for two distinct mechanisms of magnetoreception [6,7]; the magnetite-based mechanism (MBM) involving single domain or super-paramagnetic particles of biogenic magnetite [6,8,9], and the radical pair mechanism (RPM) involving a light-dependent biochemical reaction that forms long-lived, spin-coherent radical pairs. A specialized class of photopigments (i.e., cryptochromes) has been suggested to play a central role in the RPM [10-13], with the alignment of the magnetic field modulating the response of these photopigments to light [14]. Importantly, these two mechanisms are not mutually exclusive. For example, in amphibians and birds a MBM is thought to derive geographic position (“map”) information, while the light-dependent RPM provides directional (“compass”) information ([15], but see [16,17] for MBM based compass orientation in subterranean mammals). The use of a light-dependent magnetic compass, despite the presence of a magnetite-based receptor with properties arguably better suited for this task (i.e. sensitivity to the polarity of the magnetic field, ability to operate in total darkness), suggests that the light-dependent mechanism may provide more than simple directional information [18].

Behavioral studies have helped characterize the functional properties of both the MBM and the RPM [5,15,19-26]. For example, in insects, amphibians and birds, the magnetic compass has been shown to be dependent on light [23,24,27-31], and sensitive to the inclination, but not polarity, of the magnetic field [22,32]. Both results are consistent with a RPM [33-35]. In contrast, mole-rats that inhabit aphotic subterranean habitats have a MBM-based magnetic compass that operates in total darkness and is sensitive to the polarity
of the magnetic field [16]. To date, responses showing properties of both mechanisms have only been observed in animals that use magnetic cues in both the map and compass components of long-distance navigation (newts [22,36], birds [37,38] and sea turtles [39]), suggesting that these responses may receive inputs from both mechanisms, rather than the involvement of a third, distinct mechanism (see below).

A diagnostic property that distinguishes unambiguously between a MBM and RPM is sensitivity of the RPM to low-level radio frequency (RF) fields [40]. For instance, magnetic compass orientation of birds is disrupted by low-level RF fields at the Larmor frequency (i.e., the precession frequency of an electron spin in the ambient magnetic field) [41], as well as low-level RF noise, consistent with the involvement of a RPM [40]. Recent findings suggest that the sensitivity of a RPM based system may be as low as 1 nT (RF intensity), which can be in the range of anthropogenic electromagnetic noise [42]. In contrast, non-light-dependent, SMA of mole-rats was unaffected by RF fields at intensities as high as 4800 nT [43], as would be expected for a MBM.

Interest in SMA, first reported in several insects [44], is experiencing a revival in the current literature [45-50]. Spontaneous magnetic alignment is defined here as an untrained, non-goal-directed, body alignment relative to the Earth’s magnetic field (often resting or landing positions). Although the proximate and ultimate causes of SMA are not understood, it may serve a variety of important functions. For example, it has been suggested that maintaining a fixed alignment relative to the magnetic north-south axis may facilitate measurement of magnetic inclination and/or total intensity, increase the accuracy of 3-dimensional (3-D) targeting and course control, and/or help to encode spatial input from other sensory modalities [18,46,51,52].
The putative magneto-receptive molecule for a RPM, cryptochrome [53,54], has been shown to occur in photoreceptors in the pineal complex and/or retina of vertebrates, and compound eye of insects [35,55-57]. Specific classes of photoreceptors, may be specialized for sensing the geomagnetic field, e.g. the UV/violet cones in the retina of birds, extra-ocular photoreceptors mediating chromatic responses in the pineal complex of amphibians [35], and central retinula cells in the compound eyes of flies [7]. In animals, in which the specialized photoreceptors are located in the retina or compound eye, the magnetic field may be perceived as a 3-dimensional ‘visual’ pattern that appears to surround the animal and be superimposed on its surroundings ([11,14,18,58-60]; and see below). In effect, the animal would be at the center of a simple spherical coordinate system that is fixed in alignment relative to the magnetic field as it moves through the environment.

In the present study, experiments were carried out to determine if an earth-strength magnetic field influences alignment behavior of yearling snapping turtles (*Chelydra serpentina*). In particular, the experiments focused on the role that magnetic cues might play when an animal is introduced to a novel environment. Exposure to low-level RF fields was used to determine whether a RPM was involved in the SMA of turtles, and whether the conditions under which the turtles were first introduced to the testing apparatus (with or without low-level RF fields) influenced subsequent alignment responses to the magnetic field.
Results

The alignments of turtles tested in a vertical magnetic field, which does not provide directional information, were randomly distributed (S1 Fig.) indicating that no other non-magnetic (e.g. topographic) directional cues were used by the animals in the testing arena.

Testing turtles in four symmetrical horizontal magnetic field alignments (magnetic N = topographic N, E, S, W) made it possible to partition the alignment responses into topographic and magnetic components (see Material and Methods). In all treatment conditions, topographic distributions were indistinguishable from random (S2 Fig.), providing further evidence that the animals were not relying on non-magnetic cues.

Animals initially exposed to the geomagnetic field without RF and subsequently tested in the four magnetic field alignments without RF (‘RF off → RF off’ condition) showed consistent alignment relative to the magnetic field with the mean vector bearing towards magnetic north (p < 0.005, R* = 1.388, n = 18, Moore’s modified Rayleigh test, Fig. 1). When tested in the four magnetic field alignment with RF (‘RF off → RF on’ condition) these same animals failed to exhibit a consistent alignment relative to the magnetic filed (n. s., R* = 0.121, n = 18, Moore’s modified Rayleigh test). The difference between the ‘RF off → RF on’ and ‘RF off → RF off’ responses approached significance (p < 0.1, R' = 1.005, n = 18, Moore’s paired two-sample test).
Figure 1: Magnetic component of responses of individual turtles plotted relative to magnetic North (mN).

Magnetic directional preferences of turtles combined from four magnetic field alignments (magnetic north aligned in each of the four cardinal directions). Responses of turtles initially exposed to the magnetic field without RF are shown in the left column with black outer circles. Responses of turtles initially exposed to the magnetic field in the presence of the RF stimulus are shown in the right column with red outer circles. Black lines connected to black dots (labeled “RF off”) are the mean vectors showing the magnetic component pooled from the four magnetic field alignments in which the turtles were tested without RF, and the responses shown in red (labeled “RF on”) are from the four magnetic fields alignments in which the same turtles were tested in the presence of RF. Moore’s modified Rayleigh test
was used to test each distribution for non-random unimodal alignment. Dependent data, e.g., diagrams in the left column or in the right column with the same colored circles (showing data collected from the same individuals), were tested for significant differences using Moore’s paired sample test. Independent data (i.e. distributions in the same horizontal row obtained from different individuals) were tested for significant differences using the Mardia’s two-sample test.

The behavior of animals initially exposed to the geomagnetic field with RF present was remarkably different. After initial exposure to the magnetic field with RF present, animals subsequently tested in the four magnetic field alignments without RF (RF on → RF off; Fig. 1) were disoriented (n. s., R* = 0.674, n = 18, Moore’s modified Rayleigh test). However, in the four magnetic field alignments with RF present (RF on → RF on), these same animals showed consistent alignment relative to the magnetic field (p < 0.01, R* = 1.307, n = 18, Moore’s modified Rayleigh test). Interestingly, however, the direction of magnetic alignment was toward magnetic south, i.e., opposite that of the ‘RF off → RF off’ group (Fig. 1), and the distribution of magnetic responses in the two groups of turtles that were pre-exposed and tested in the same RF condition (‘RF off → RF off’, and ‘RF on → RF on’) were significantly different (p < 0.001, U = 0.42, n = 18, Mardia’s two-sample test, Fig. 1).

Discussion

Spontaneous magnetic preferences are known for many different taxa, from arthropods to mammals [50]. In vertebrates, these behaviors typically consist of unimodal or axial responses aligned near the magnetic north-south axis [50]. In the present experiments, directional responses of snapping turtles were tested in four symmetrical magnetic field alignments, which made it possible to isolate the component of the behavior that showed a
consistent alignment relative to the magnetic field (see Methods). In the absence of any influence of RF fields (‘RF off → RF off’ condition), the turtles’ directional responses showed a consistent northward alignment relative to the magnetic field, providing the first evidence for SMA in a reptile.

Previous experiments with migratory birds have shown that exposure to a RF field at the Larmor frequency at intensities as low as 15 nT can abolish magnetic compass orientation [61]. In the present experiments, turtles that were initially exposed to the testing apparatus without RF, failed to exhibit a consistent alignment relative to the magnetic field when subsequently tested in four symmetrical magnetic field alignments with RF present (‘RF off → RF on’ condition). Because the energy of interaction of the radio frequency field used in the present experiments (1.43 MHz at 30-50 nT) with an unpaired electron is many orders of magnitude below the thermal noise floor [40], this result is compelling evidence for a quantum process, such as the RPM, underlying the turtles’ SMA responses.

In contrast to turtles introduced to the testing chamber without RF and tested with RF (‘RF off → RF on’ condition), turtles introduced to the testing chamber in the presence of RF showed consistent alignment relative to the four magnetic field alignments when RF was present (‘RF on → RF’ on condition). However, they were disoriented when tested in the same four field alignments without RF (‘RF on → RF off’ condition). Moreover, the directional response of turtles in the ‘RF on → RF on’ condition (towards magnetic south) was opposite to that of turtles in the ‘RF off → RF off’ condition (towards magnetic north). Taken together, these findings indicate that RF fundamentally alters, but does not abolish, the directional information that the turtles obtained from the magnetic field. These findings also show that the presence or absence of RF when an animal is first introduced to novel
surroundings influences its subsequent response to the magnetic field, i.e., turtles only showed a consistent alignment relative to the magnetic field in the same RF condition in which they were first introduced to the testing apparatus.

Our findings are consistent with evidence from animals as different as flies and mice that a complex, 3-D pattern is generated by the RPM [34]. In studies in which animals are trained to orient in a particular heading relative to the magnetic field [24,51,62,63], and/or have been shown to calibrate the magnetic compass relative to a global reference system (e.g. [64]), failure to ‘recognize’ an altered pattern of magnetic input might have prevented the animals from orienting in an RF condition that differed from the one in which they had learned, or calibrated, the compass response [11]. However, because yearling snapping turtles in the present study exhibited spontaneous (i.e., unlearned and uncalibrated) alignment relative to the magnetic field (Fig. 1), neither of these explanations can account for the dependence of the effects of RF exposure on initial experience in the novel testing environment.

Instead, the findings suggest that yearling turtles formed a rapid association between the novel surroundings and RPM response pattern, which differed depending on the initial RF exposure. This apparently spontaneous association seems to have a ‘sensitive period’, when the turtle is first introduced to novel surroundings during which this association is formed. The association lasted for a minimum of several hours (the duration of testing in the present experiment), and was unaffected by subsequent exposure to a pattern of magnetic input that differed depending on the RF-exposure condition. Further experiments are needed to determine whether the association persists over longer periods of time and if this
association is retained when an individual is removed and subsequently returned to the surroundings.

Under natural conditions turtles would never experience a change in the pattern of magnetic input. Therefore, if turtles form a similar association of magnetic cues with the surroundings each time they encounter unfamiliar habitat, as seems likely, the same pattern of magnetic input would be associated with multiple sites/localities. This would be expected from a sensory input that functions as a global reference frame, helping to place multiple locales (i.e., multiple local landmark arrays) into register to form a global map of familiar space.

The turtles response to magnetic cues in the presented experiments parallels the earlier findings of Collett and Baron [65] who showed that honeybees revisiting a familiar environment align themselves relative to the magnetic field so that they faced a radially-symmetrical visual landmark from a consistent magnetic direction. Based on these findings, as well as their earlier research [66,67], the authors suggested that magnetic input provides honeybees with a coordinate system that simplifies spatial pattern recognition by aligning the animals so that “the pattern of landmarks imaged on their retina matches the pattern stored on previous visits to that place“ [65]. Perhaps, as suggested by [18], the pattern of magnetic input from the RPM is ‘mapped’ onto the local environment analogous to taking a mental ‘snap shot’ of the novel surroundings with the 3-D ‘grid’ superimposed. Although much remains to be learned about the role of the magnetic field in encoding spatial information in vertebrates, evidence that mice require only two brief (< 60 sec) trials to encode the magnetic direction of a submerged platform in a ‘plus’ water maze task [51] clearly indicates the need
for further investigation of the role of magnetic cues in organizing spatial information in novel surroundings.

Given the likelihood that exposure to low-level RF fields directly affects the RPM [40,61], the findings reported here place important constraints on the properties of the underlying biophysical mechanism (Peter Hore, personal communication). The ‘reference-probe’ radical pair design proposed by Ritz et al. [68], in which one member of the radical pair is devoid of hyperfine interactions, would predict a strong resonant response to the Larmor frequency (used in this study) that would reduce the anisotropy of the response of the radical pair and thereby destroy the directional information required for magnetic orientation [69]. However, our results suggest that RF is modifying the response of the RPM, altering rather than eliminating the resulting directional information. Such an effect could arise from a more complex radical pair design in which both radicals have hyperfine interactions. For instance, FAD-tryptophan could be molecules involved in such a mechanism (as originally proposed by Ritz et al. [14]). Importantly, however, in order for RF fields at such low intensities to affect the radical pair, the spin-relaxation would need to be very slow, on the order of several milliseconds, which is unprecedented in known radical pair systems (Peter Hore, personal communication). Moreover, a FAD-tryptophan radical pair would be expected to exhibit peaks of sensitivity to RF fields outside the Larmor frequency range, as may be the case in migratory birds [42,61].

In conclusion, the present study demonstrates spontaneous magnetic alignment by a reptile similar to that reported previously in other vertebrates, and shows the sensitivity of SMA to low-level RF fields at the Larmor Frequency. On the one hand, the findings confirm how little is actually known about the biophysical process underlying the RF-dependent
magnetoreception mechanism, despite evidence that this type of mechanism may be present in animals as diverse as birds [61], epigeic rodents [34], reptiles (this study), amphibians [23], and some insects [70]. What is arguably the most important finding from these experiments, however, is that in addition to the well-studied use in goal-directed orientation, the magnetic field appears to play an important, yet poorly understood role, in encoding spatial information in the animal’s immediate surroundings.

**Material and Methods**

**Turtle collection and husbandry**

From April-July 2011, eggs were collected and incubated from gravid female *C. serpentina* captured along the South River and from nearby sites along the Middle River (both Virginia, USA) using baited hoop traps (see [71,72] for more information on trapping, egg collection and incubation). Once the incubated eggs hatched in Aug-Sept, individual hatchlings were housed in plastic, opaque Ziploc® containers (591 ml; S.C. Johnson & Son, LLC, Wisconsin, USA) filled with 150 ml dechlorinated water placed in an environmental chamber controlled at 25°C (observed temperature 25.46 ± 0.01°C) with a 12:12 light:dark cycle. Beginning in Oct, we induced hibernation by gradually (1°C increments) reducing temperature each week to 4.5°C. After 5 months in hibernation, temperatures were gradually increased to 25°C. The turtles were tested within one month of emergence in May 2012. All hatchlings were released at the end of the study at the site of maternal origin.

**Testing procedure**

Testing trials took place in a double-walled testing building to minimize external sources of variation, such as vibration and sound cues. Additionally, the testing apparatus
was located in a grounded faraday cage, and all power lines going into the enclosure were equipped with EMI/RF filters (Dearborn, 1JX2459) to reduce background electromagnetic noise. The testing apparatus (including the testing arena and surrounding magnetic coils; see below) was mounted on a ~400 kg sandbox supported by a ~10 cm layer of compressed fiberglass insulation to further reduce substrate vibrations.

In each testing session, 6 yearling snapping turtles were placed in individual testing chambers (Fig. 2) inside a testing apparatus surrounded by a double-wrapped, cube surface coil [73]. Full-spectrum light was provided by an overhead light source centered above the testing arena, but outside the magnetic coils. The light passed through two frosted glass diffusers before reaching the testing arena. Turtles were tested inside individual testing chambers consisting of a Pyrex bowl, a PVC cylinder inside the bowl that visually isolated each subject, and a milky white Plexiglas diffuser placed on top of each testing chamber (Fig. 2). The chambers were placed on the floor of the testing apparatus made from a clear glass plate covered by gray fiberglass insect screen (light reflection from the screen prevented the animals from seeing through the bottom of the glass bowls and, therefore, from responding to any visual asymmetries beneath the arena).
Turtles were tested in a Pyrex bowl with water that was ~1 cm deep so the turtle’s shell was not completely submerged. The Pyrex bowl, PVC surround, and overhead diffuser provided uniform visual surrounding.

Each test day, all 6 turtles were exposed to nine consecutive treatments that were determined in a pseudorandom order. Turtles exposed to RF in the first of the 9 testing conditions (3 of the 6 testing days) were exposed to RF during the 20 min acclimation period prior to the start of testing. Experimental treatments were as follows: vertical field only (no directional magnetic cues); mN = topographic north, south, east and west with RF present; mN = topographic north, south, east, and west with RF absent. Each individual was exposed to each of the 9 treatments once for at least one hour. One hour of video was recorded in each condition, and only the last 40 minutes were used for the body alignment analysis. Animals were not removed from the testing chambers between treatments; however, there was always at least a 20-minute acclimation period in each new treatment condition, before observations of body alignment were recorded.
All changes in magnetic field conditions and RF conditions were produced from outside the Faraday cage. Other than the electromagnetic changes, the turtles were not exposed to disturbance of any kind nor did the experimenter enter the Faraday cage that surrounded the testing apparatus during the entire sequence of treatment conditions.

The behavior of the turtles was recorded from underneath the testing arena through the glass bottoms of the testing bowls by a video camera located below the shielded enclosure. The body axis directions of each of the turtles were measured on 36 frames (0.9 frames per minute, 40 minutes). All measurements were done following a double blind procedure. Video recordings were given arbitrary numbers using a random sequence by a person not involved in the experimental design or behavioral measurements (M.P.). A single observer (L.L.) then measured directions for each of the videos in random order without knowing the identity of the turtles, treatment, or test day.

The posture of each animal was recorded and categorized for each frame using the categories “neutral posture”, “probably moving – direction not measureable”, or “crawling up against the wall”, measurements in the latter two groups were excluded from the analysis. Overall, 3.5% of the measurements were excluded (409 out of 11664). All measures were obtained using a double blind protocol from a single person (described above), to avoid observer biases.

**The magnetic field manipulations and radio frequency interference**

The animals were tested in four different alignments of an earth-strength field (51.24 ± 0.06 µT, inclination angle 64.3° ± 0.6°), with magnetic north aligned to topographic north, east, south or west. The magnetic fields were generated by a pair of horizontally aligned,
double-wrapped Rubens coils [73,74]. The magnetic field components generated by the two coils were controlled by reversing the direction of current flow through one of the two wraps [5,74]. For the vertical field condition, the horizontal field component was cancelled resulting in field strength of 46.78 µT (the residual of the horizontal component was 0.7 µT, towards 348°).

The radio frequency field was generated using a signal generator (Agilent, model 33250a), amplifier (Amplifier Research Associates, model 10A250), and an antenna consisting of a single, horizontal loop of wire surrounding the testing apparatus. The angle between the RF field and the magnetic field (26 ± 2°) was the same in all four horizontal alignments of the earth-strength magnetic field, and similar to that shown to disorient magnetic compass orientation in an earlier study of migratory birds [61]. The AC field was set to approximate the Larmor frequency for the ambient magnetic field strength (1.430 MHz). The intensity was measured at the location of each testing chamber and varied from 30 to 52 nT, depending on the location. Although variation in RF conditions were recorded across the testing apparatus (higher RF intensities occurred around the periphery of the cluster of testing chambers, i.e., in chambers located closer to the radio antenna), each turtle remained in the same testing chamber, and therefore, the same absolute position throughout the experiment, and thus experienced a constant RF field intensity in the treatment conditions in which it was exposed to the RF stimulus.

Testing in four symmetrical magnetic field alignments (magnetic north aligned to each of the cardinal compass directions), both with and without RF exposure, made it possible to partition the directional responses of individual turtles into magnetic and non-magnetic (topographic) components [62]. For example, to determine the topographic
component of one of the turtles in the absence of RF exposure (i.e., ‘RF-off’), the directional responses from the four testing conditions were pooled without taking into account the magnetic field alignments. The vector sum of all individual responses from the four testing conditions pooled in this way provides a measure of the turtle’s response to any consistent non-magnetic (i.e., topographic) cues present in the testing chamber. To determine the magnetic component of the turtle’s response, the individual responses obtained in the same four testing conditions were pooled after first being rotated so that the directions of magnetic north coincided. The vector sum of all bearings of one individual pooled in this way provides a measure of the turtle’s response to the alignment of the static magnetic field. Similar analyses of the directional responses in the four field conditions in which the turtle was exposed to the RF field was then carried out to determine the topographic and magnetic components of response when exposed to the Larmor Frequency field (‘RF on’).

The magnetic and topographic responses of the individuals were then summed for each of the conditions: 1) ‘RF off → RF off’, 2) ‘RF on → RF off’, 3) ‘RF on → RF on’, 4) ‘RF off → RF on’, 5) ‘RF off → vertical field (RF off)’, 6) ‘RF on → vertical field (RF off)’.

All second order circular distributions of topographic and magnetic responses (each individual presented by a single vector) were analyzed with a second order Moore’s modified Rayleigh-test [75]. For comparisons of paired directional observations (‘RF off → RF off’ with ‘RF off → RF on’ and ‘RF on → RF off’ with ‘RF on → RF on’) the Moore’s paired sample test was used. For independent comparisons (‘RF off → RF off’ with ‘RF on → RF on’, ‘RF off → RF off’ with ‘RF on → RF off’, ‘RF off → RF on’ with ‘RF on → RF off’, ‘RF off → RF on’ with ‘RF on → RF on’) a Mardia’s two-sample comparison test was
applied [76]. All analyses, including the 95% confidence intervals, were calculated in Oriana 4.0.

**Ethics Statement**

Animal collections were permitted by the State of Virginia (Permit #: VA department of Game and Inland Fisheries #035981). The presented research was approved by the Institutional Animal Care and Use Committee of the Virginia Tech (#09-080-FIW, Amendment #2). Experiments were purely observational; the animals were released unharmed to their original habitat at the conclusion of the experiments.

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**References**


Supporting Information

S1 Figure: Alignment in vertical magnetic field.

The results of the vertical field treatments (no RF was applied during this treatment). The results show random orientation for the days with (red outer circle) and without (black outer circle) RF acclimation. Moore’s modified Rayleigh test was used to test each distribution for significant unimodal alignment.
S2 Figure: Topographic component of responses.

The results combining the four magnetic field treatments (N, E, S, W), however, analyzed with regard to the topographic (geographic) north, showing that there is no topographic bias in the distribution. RF off acclimated animals are shown with a black outer circle, RF on acclimated animals with a red outer circle. Moore’s modified Rayleigh test was used to test each distribution for significant unimodal alignment. Dependent data (alignment of same individuals in the two treatments RF off and RF on) were tested for significant differences using the Moore’s paired sample test. Independent data were tested for significant differences using the Mardia’s two-sample test.
3. High levels of maternally transferred mercury disrupt magnetic responses of snapping turtle hatchlings (*Chelydra serpentina*)

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*In preparation for submission*

**Abstract**

A wide range of animals uses the Earth’s magnetic field for a variety of spatial behaviors, ranging from long-distance migratory orientation and homing to non-goal directed behaviors such as spontaneous magnetic alignments (SMA). Mercury is a harmful pollutant most often generated from anthropogenic sources that can bio-accumulate in animals over a lifetime and is known to be neurotoxic to fish, wildlife, and humans. Mercury can be also transferred from mother to offspring. Because mercury could interfere with the use of magnetic cues in spatial behavior, either through a generalized effect on navigation circuits in central nervous system or through a direct effect on the primary sensory mechanism(s) responsible for magnetoreception, we tested potential effects on a magnetic response in yearling snapping turtles in addition to standard performance measures. Turtle yearlings from a reference site and a site with high natural occurring mercury levels were tested simultaneously, while the
experimenter was blind to their real IDs. Turtles were exposed to a symmetrical set of earth-strength magnetic field alignments in combination with and without the addition of low-level radio-frequency (RF) fields, a diagnostic tool used to investigate the light-dependent magnetic compass mediated by a radical pair mechanism (RPM), for a total of 8 treatment conditions. Additionally, turtles were tested in a vertical magnetic field that did not provide any magnetic directional information. Reference turtles showed RF dependent magnetic alignment responses along the north-south axis (published previously), consistent with many vertebrate SMA responses. Although mercury exposure did not influence any of the standard performance measures commonly used in ecotoxicology studies, such as righting response and maximum velocity, the responses of the turtles to magnetic cues were abolished. There is growing evidence, including from the responses of turtles from the reference site in the present study, that magnetic cues may not only be involved in long-distance, goal-directed movements, but also play an important role in organizing spatial information from the animal’s immediate surroundings when it encounters unfamiliar habitat. Spatial orientation is critical to the early stages of turtles, including the migration of hatchlings to the water and orientation inside the water column. Future research is needed to determine the threshold for effect of mercury on SMA of yearling turtles and whether mercury exposure compromises movement behavior of adult turtles, such as nesting migrations and nest site selection of adult females.

Introduction

Magnetic sensing has been shown in a large variety of taxa, including turtles [1-3]. While most studies have focused on the use of magnetic cues for goal-oriented movement, many animals also show spontaneous magnetic alignment (SMA). This includes many vertebrate taxa in which the body axis is aligned in a consistent relation to the Earth’s
magnetic field (e.g. cattle [4], foxes [5], dogs [6] and trout [7]). Although, the mechanisms underlying SMA are still under debate, there are two leading hypotheses to explain magnetoreception. 1) Magnetite-based mechanism (MBM), involving single domain or superparamagnetic particles of magnetites [8] and 2) Radical pair mechanism (RPM) in which the Earth’s magnetic field influences spin states of uncoupled electrons potentially altering the response of specialized photoreceptors to light [9]. Both mechanisms appear to be present in a single animal. MBM is thought to provide a magnetic map reading (in at least some vertebrates) and the RPM is thought to provide a magnetic compass [10-12], but see also Thalau et al. [13].

Magnetoreception is important in many aspects of spatial behavior, and therefore compromising magnetic cue use could have negative effects on animal fitness. Magnetic cues are used as a compass in local and long-distance movements [14-19]; as an important component of ‘navigation’ providing a magnetic map [20,21]; as a global reference reducing errors in path integration [22,23] and potentially as a spherical coordinate system helping to encode the immediate visual surrounding [15,23]. 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 other vertebrate SMA responses [15]. The magnetic response was sensitive to low-level radio frequency (RF) fields at the Larmor frequency, providing compelling evidence for a RPM based magnetic compass [24]. Interestingly, RF did not simply disrupt the magnetic response instead RF altered the response. This affect was dependent on the first experienced RF exposure in the testing environment (‘pre-exposure’).
While animals that were pre-exposed to RF off and tested under RF off (RF off → RF off) showed an alignment towards magnetic north, animals pre-exposed to RF on and tested under RF on (RF on → RF on) magnetically aligned towards south. In contrast when pre-exposure RF differed from testing RF (RF off → RF on and RF on → RF off), animals did not show a magnetic response. These results are consistent with the earlier proposal that animals take mental ‘snap shots’ with a perceived magnetic grid overlaid when introduced to new environments [23], similar to retinotopical ‘image matching’ in insects, which can also be based on a magnetic compass [25,26].

Mercury (Hg) is a very harmful pollutant, which is used and released into the environment by humans [27]. Mercury is widespread, deleterious to wildlife and produced by anthropogenic sources such as coal burning power plants [28,29]. In aquatic habitats mercury is methylated into methylmercury (MeHg), which is the more toxic molecule [30,31]. It can bio-accumulate over an individual’s lifetime and maternally transfer [32]. A variety of studies have shown negative mercury effects on reproduction [33-36] and behavior [37,38]. Reproductive behavior in aquatic and semi-aquatic animals are often linked to extended movement periods, i.e. amphibians and fish migrating to their spawning grounds [39,40], or turtles migrating on land in order to find suitable areas to incubate their eggs [41]. In order to accomplish such challenging spatial behaviors animals use a complex array of sensory inputs; one cue many migrating animals use is the Earth’s magnetic field [3]. Here we tested whether a common environmental pollutant disrupts the spontaneous magnetic responses of hatchling snapping turtles. Siblings of the snapping turtles used in the magnetic alignment experiment were also subjected to standard performance tests (righting response and movement speed), regularly used to measure toxicity effects. We provide evidence for impacts of mercury on spatial behavior of turtles and discuss this in relation to their ecology and life history.
Material and Methods

Turtle collection and husbandry

For specific details concerning egg collection and husbandry refer to [15]. We collected eggs from females nesting in uncontaminated reference sites along the 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. 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 [42] for additional information regarding Hg analysis). In total, we tested multiple measures of performance of a single hatchling from 12 reference and 12 contaminated clutches. 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. [43]) within an environmental chamber 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. Once conditioned, we returned individuals to their respective housing container.

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 individual 591-ml opaque Ziploc® containers filled with 2 cm dry, coarse sand. We labeled each container with the corresponding hatchling’s identity, and arranged the containers in a 2x3 grid that allowed us to test six individuals simultaneously. We suspended a digital video camera above the arena grid to record each experimental trial. We randomly placed individual hatchlings into one of the six experimental arenas, allowed them to acclimate for two minutes, and then inverted each individual on to their carapace. We 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, we considered the trial completed and returned each turtle to its respective housing container. We then provided individuals two minutes of rest before
repeating the trial twice for each hatchling, resulting in three righting response trials per individual.

We characterized righting response by three components of turtle righting performance: 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. We reviewed righting response videos using Adobe® Encore CS5 software (Adobe Systems Incorporated, CA, USA).

**Magnetic alignment testing procedure**

For detailed description of the methodological procedure see [15]. Magnetic fields were generated by double-wrapped Rubens coils ([44,45], which produced a mean field strength of $51.24 \pm 0.06 \, \mu T$ (the vertical field had a strength of $46.78 \, \mu T$), allowing us to align the magnetic north to topographic (= geographic) north, east, south and west. The RF intensities ranged from about $30 \, nT$ to $52 \, nT$ (1.430 MHz, the local Larmor frequency), based on the location of the testing chamber in relation to the RF coil.

The animals were tested for magnetic alignment in individual chambers; each consisted of a Pyrex bowl, a PVC tube and a frosted glass diffuser (see previously published methods [15]). On each test day twelve animals were tested simultaneously, six of which were controls, i.e. no mercury exposure (published earlier [15]), and six animals with high mercury contamination. Each animal experienced all four magnetic field alignments (with and without introduced radio frequency) and the vertical field in a pseudo-random order. This resulted in 9 different conditions, which were presented to the animals at least for one hour.
The trials were recorded by a camera and from each video the last 40 minutes were used for further analysis, 36 frames (0.9 frames per second) out of each recording were analyzed.

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 frames were discarded (824 out of 23328) due to this reason. All measurements were collected with the experimenter being blind to the identity of the individuals (mercury or reference animal) turtle identities were revealed after data analysis was completed.

**Statistical Analysis**

We used SAS 9.2 (SAS Institute, Inc, Cary, NC, USA) for all statistical analyses regarding the performance measures and assessed significance at $\alpha \leq 0.05$. When appropriate, we log10-transformed 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. Additionally, in order to understand 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 condition (RF off → RF off, RF on → RF on, RF off → RF on, RF on → RF off and the vertical field). The resulting distributions were tested for unimodal distribution using the Moore’s modified Rayleigh-test [46], paired observations were tested for difference using the Moore’s paired sample test and independent observation were tested for difference using the Mardia’s two-sample comparison test [47]. For all circular statistics Oriana 4 was used. See Landler et al. [15] for specific details on 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 (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 ($F_{1, 23}=0.64, p = 0.43$, see also figure 1) or hatchling righting response ($F_{3, 20}=0.95, p = 0.44$, see also figure 1).
Figure 1: Results of performance tests.

Comparison of hatchling righting response components (A,B,C) and locomotor performance (D) of individuals produced by females collected from either reference or Hg contaminated sites along the Middle and South Rivers, respectively (Waynesboro, VA, USA). Bars represent means ±1 S.E.
In an earlier paper [15], we showed that turtles from the reference site exhibit constant alignments relative to the magnetic field when tested in the same RF condition that 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); while turtles from the reference sites were disoriented in an RF condition that differed from that initially experienced in the testing apparatus (i.e. in the RF off → RF on, and in the RF on → RF off conditions, data not shown). In contrast, the mercury-contaminated animals did not show consistent magnetic orientation, in either condition (figure 2 compares the distribution of response in the RF on → RF on and RF off → RF off condition and see Landler et al. [15]). The distribution of alignments of controls collected at the reference site were significantly different from those of the mercury-contaminated animals when comparing ‘RF off → RF off’ conditions (U = 0.276, p < 0.01, Mardia's two sample test, figure 2) and approached significance in the ‘RF on → RF on’ conditions (U = 0.177, p < 0.1, Mardia's two sample test, figure 2).
Figure 2: Comparison between control and mercury contaminated yearling snapping turtles.

The left panel shows the circular distributions of control 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-random unimodal alignment. Data were tested for significant differences using the Mardia’s two-sample test.
When analyzed in relation to topographic north (i.e. ignoring the alignment of the magnetic field) controls did not show consistent non-magnetic alignment that would indicate reliance on non-magnetic cues. The distribution of alignments of animals collected from the mercury site was significantly aligned relative to topographic north in one of four testing conditions (‘RF off → RF on’ condition; R* = 1.308, p < 0.01, Moore’s modified Rayleigh test, figure S1), in all other conditions, including the vertical field condition, the topographic response did not reach significance (figure S2 & S3).

**Discussion**

Our findings strongly suggest that maternally transferred mercury can disrupt magnetic responses in yearling snapping turtles. Considering the potential importance of magnetic cues in spatial orientation and the various orientation challenges also hatchling turtles are facing, the impediment of spatial abilities is likely to have survival consequences. For instance, turtle hatchlings have to move rapidly towards the water after hatching on land and then successfully orient them in the water column after reaching the water [48-53].

In contrast to yearling turtles from the reference site that exhibited RF dependent magnetic alignment responses [15], turtles from the mercury contaminated site failed to exhibit consistent alignment responses relative to the Earth’s magnetic field. Also, reference animals appeared to use the pattern of response produced by the magnetic field in order to encode the novel environments, however, such ability was lacking in mercury-contaminated turtles. Interestingly, we did not find any differences between reference and mercury-contaminated turtles when comparing righting response and movement speed.
The non-magnetic (i.e. topographic) alignment of mercury exposed turtles in one of the four conditions suggests that in the absence of a response to magnetic cues the turtles may have been responding to another directional cue, unrelated to the magnetic field, however, the potential source is unknown. Also, the lack of a magnetic response is unlikely to be based on a mercury influence on basic motor skills, as tests of general motor skills were unaffected by mercury contamination. This raises the interesting possibility that there are more specific effects on magnetic behavior, maybe even at the receptor level.

Mercury could potentially interfere with both proposed magnetoreception mechanisms, by binding to either magneto-sensitive compound. First, radio-frequency dependent magnetoreception, as shown in the current study, is very likely based on a RPM or a similar quantum process [24]. The putative receptive molecule for a RPM is cryptochrome [54-57], a molecule best known for its role in circadian rhythms [58]. Recently, it has been shown that a Zinc ion serves as a cofactor in cryptochromes, serving to modulate cryptochrome-period interactions [59]. Substitution of mercury for the Zinc cofactor [60] could disrupt the interactions of cryptochrome with a signaling partner as it occurs with cryptochrome binding to period. Second, if the observed SMA were the result of two interacting magnetoreceptor systems (RPM and magnetite based mechanism), mercury could interfere by binding to magnetite, which has a high affinity for Hg [61,62]. Alternatively, the effects could not be specific to the magnetoreceptor system, but constitute broader physiological impacts. For example, mercury can negatively affect vision [63,64] and also learning and memory [38,65] and therefore disrupt the potential association between the visual pattern produced by the magnetic field and the visual surrounding.
Mercury levels at the contaminated sites in this study were very high in relation to other examples of mercury contamination in natural populations [63]. Further research studying the effects of lower concentrations of mercury is needed in order to determine the threshold for impacts on wildlife populations. Likewise, because mercury exposure was entirely of maternal origin, additional work is needed to determine if dietary exposure to Hg could produce similar effects. Finally, data are needed to determine if the observed effects of mercury on magnetic behavior are long lasting, possibly influencing spatial behavior at other life history stages. Such questions could be explored by testing, for instance, homing performance of adult snapping turtles displaced from reference vs. Hg 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 animals immediate surrounding [16,23,66,67]; i.e. animals can use the magnetic field as a stable reference to organize spatial information from a multiple sensory inputs. Therefore, magnetic cues should be viewed as a fundamental component of the orientation cues turtles can, disruption of such ability is likely to affect the turtle’s spatial abilities as a whole. The herein presented results are only a first attempt to understand the multiple and complex influences maternally transferred mercury could have on natural turtle populations. Using assays besides testing gross motor performance, including spatial learning abilities and responses to different orientation cues, might unravel the profound effects of environmental toxicants on wildlife populations.

References


**Figure S1: Topographic component of responses of individual turtles with high maternally transferred mercury.**

The results combing the four magnetic field treatments (N, E, S, W), however, analyzed with regard to the topographic (geographic) north, showing that there is no topographic bias in the distribution. RF off acclimated animals are shown with a black outer circle, RF on acclimated animals with a red outer circle. Moore’s modified Rayleigh test was used to test each distribution for significant unimodal alignment. Dependent data (alignment of same individuals in the two treatments RF off and RF on) were tested for significant differences using the Moore’s paired sample test. Independent data were tested for significant differences using the Mardia’s two-sample test.
Figure S2: Magnetic alignment of individual turtles with high maternally transferred mercury, plotted relative to magnetic North (mN).

Magnetic directional preferences of turtles combined from four magnetic field alignments (magnetic north aligned in each of the four cardinal directions). Responses of turtles initially exposed to the magnetic field without RF are shown in the left column with black outer circles. Responses of turtles initially exposed to the magnetic field in the presence of the RF stimulus are shown in the right column with red outer circles. Black lines connected to black dots (labeled “RF off”) are the mean vectors showing the magnetic component pooled from the four magnetic field alignments in which the turtles were tested without RF, and the responses shown in red (labeled “RF on”) are from the four magnetic fields alignments in which the same turtles were tested in the presence of RF. Moore’s modified Rayleigh test was used to test each distribution for non-random unimodal alignment. Dependent data, e.g., diagrams in the left column or in the right column with the same colored circles (showing data collected from the same individuals), were tested for significant differences using Moore’s paired sample test. Independent data (i.e., distributions in the same horizontal...
row obtained from different individuals) were tested for significant differences using the Mardia’s two-sample test.

**Figure S3:** Alignment in vertical magnetic field alignment of individual turtles with high maternally transferred mercury.

The results of the vertical field treatments (no RF was applied during this treatment). The results show random orientation for the days with (red outer circle) and without (black outer circle) RF acclimation. Moore’s modified Rayleigh test was used to test each distribution for significant unimodal alignment.
4. Ectosymbionts alter spontaneous responses to the Earth's magnetic field in crustaceans

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Abstract

We have long known that a wide variety of animals use the Earth’s magnetic field as a cue for directed movement and other spatial behaviors. Even when not undergoing long-distance movements, animal sensitivity to the Earth’s magnetic field is evident in the tendency for stationary animals in many taxa to align their body axes parallel to the magnetic field lines, i.e. spontaneous magnetic alignment (SMA). Here we show that crayfish exhibit SMA, the first demonstration of this response in a crustacean. Interestingly, however, crayfish’s SMA is altered by interactions with an obligate ectosymbiont. In controlled laboratory experiments in which we varied the alignment of the magnetic field, crayfish with moderate densities of symbionts aligned their body along the NE-SW magnetic axis, similar to the SMA shown previously in a variety of vertebrates and invertebrates. However, high symbiont densities resulted in a loss of the spontaneous magnetic response and a switch to non-magnetic or “topographic” alignment disregarding the magnetic field. This result was initially established in a study of crayfish with un-manipulated symbiont densities, and then confirmed in an
experimental study of crayfish with manipulated symbiont densities. Remarkably, however, all symbionts were removed from the crayfish, they switched to a different type of magnetic alignment response, i.e., bi-axial orientation along the “anti-cardinal” compass directions. These changes in behavior (magnetic bi-axial → magnetic axial north-south → non-magnetic orientation) coincide with shifts in fitness effects of the annelid worms on crayfish hosts from beneficial at moderate densities (mutualism) to harmful at high densities (parasitism). Our results indicate that symbionts can influence magnetically structured spatial behavior of their hosts in a complex host-symbiont interaction. We discuss how the density dependent effects might be related to spatial behaviors of the host that could increase or decrease transmission rate, potentially leading to a fitness benefit for the host and the symbionts.
Introduction

Growing evidence suggests that magnetic field sensitivity is virtually ubiquitous among motile animals and plays a fundamental role in organizing spatial behavior (1). Representatives from all vertebrate classes (2, 3) and invertebrates such as arthropods (4) use Earth’s magnetic field to provide directional and spatial information for movement across multiple spatial scales. Behavioral responses to magnetic cues likely reflect selection acting on spatial behaviors that increase individual fitness and explanations for magnetically structured behavior have squarely focused on this perspective (5, 6). However, the spatial behavior of animals is often altered by internal and external symbionts as either an incidental by-product of infection (7), or as a form of host manipulation which increases symbiont fitness often at a cost to host fitness (8). Most, if not all animals host myriad symbionts. Therefore, without consideration of how symbiotic interactions influence magnetically structured spatial behavior, our understanding of magnetic sensing and the effects of symbiosis remain incomplete.

The utility of magnetic sensing is not limited to goal-directed orientation, such as migration, but may also be involved in helping to organize and encode spatial information from other sensory modalities in an animal’s immediate surroundings (1, 9, 10). Consistent with the use of magnetic cues to structure the local environment, a variety of animals have been shown to spontaneously align their body axis with the Earth’s magnetic field, i.e., to exhibit spontaneous magnetic alignment (SMA) (11). We use SMA to describe non-goal oriented and untrained alignment of the animal body axes with magnetic fields (12, 13) and may offer fitness advantages by providing a global and stable reference for local spatial behavior (1, 10, 11). For instance, SMA might function as a rangefinder that increases accuracy of short-range predatory attacks, as it has been suggested for foraging foxes (14). Additionally, SMA offers advantages to researchers over other magnetic behaviors for
experimental study of magnetic sensing. Unlike goal-oriented movements such as homing and magnetic compass responses (15, 16), studies examining SMA do not require complex behavioral training, and therefore show an innate magnetic compass response, likely giving insights into the fundamental spatial perception and behavior.

The effects of symbionts on host spatial behavior are well-known and are the result of multiple interacting mechanisms. These mechanisms may be categorized into three types: selection acting on the host, selection acting on the symbiont, and bi-products of host-symbiont interactions. For example, fish plagued by ectoparasites will seek cleaning stations inhabited by mutualistic partners that remove harmful ectoparasites (17). This example illustrates two important drivers of symbionts on host fitness and resultant behavior: first, potential negative effects of ectoparasites parasites and secondly the benefit of interacting with cleaner species (18). But changes in host behavior may also reflect selection acting on symbionts to manipulate their hosts and increase transmission (8). Examples include lancet liver flukes (*Dicrocoelium dendriticum*) that cause ants to climb blades of grass, thereby increasing the likelihood of transmission to grazing animals; hairworms (*Paragordius tricuspidatus*) that alter the reaction of their insect hosts to light and water in order for the worms to be released in water to continue their life cycle (19); and protists (*Toxoplasma gondii*) that cause rats to seek, rather than avoid, the source of cat odors and may thereby increase the likelihood of transmission (20). Lastly, some effects of symbionts on host behavior may be purely incidental with no apparent selective advantage for either host or symbiont. For instance, fleas change mammalian spatial behavior by inflicting distracting sensory stimuli which alter the movement of their hosts in relation to resources or potential predators (7). Regardless of causality, these examples clearly show that animal spatial behavior is often linked to and influenced by symbionts, presumably because of the importance of spatial behavior in transmission. In this study, we provide the first evidence for
SMA in a crustacean. Moreover, we experimentally tested for the effects of external annelid symbionts (*Cambarincola ingens*) on freshwater crayfish (*Cambarus sciotensis*) magnetic behavior.

Astacoidean crayfish throughout the Northern Hemisphere are hosts to a monophyletic clade of obligate ectosymbiotic worms (Annelida: Branchiobdellida) (21). We chose this system to explore symbiont effects on magnetic behavior for four reasons. 1) Branchiobdellidan presence and density (number of symbionts on one host) are easily manipulated and thus provides an excellent model system for experimentally investigating the effects of symbionts on host behavior (21, 22). 2) Branchiobdellidans have complex fitness effects on their hosts. Moderate densities of branchiobdellidans can increase crayfish growth and survivorship by cleaning harmful epibiotic accumulations from their host (23, 24). At high branchiobdellidan density host growth is decreased (25), at least in part due to facultative parasitism. 3) Branchiobdellidans provide sensory input that elicits changes in host behavior that likely reflect co-evolved feedbacks between host and symbiont. For instance, branchiobdellidan abundance on crayfish can be reduced by regulatory grooming of the host (21, 26), even single worms can alter grooming behavior, e.g. in a branchiobdellidan intolerant host species (27). Tolerance to branchiobdellidan worms can also change ontogenetically, which seems to correspond to a switch of worm induced grooming activity; while small crayfish are intolerant to even single worms large crayfish are tolerant to them and do not change their grooming response even with high number of ectosymbiotic worms (22 and Skelton et al. unpublished). This switch in the behavioral response might reflect a changing cost-benefit ratio of the symbiotic interaction; while small crayfish are molting fast and branchiobdellidan worms therefore might consume more host tissue instead of cleaning epibionts off the crayfish, larger crayfish are benefiting from the cleaner function (21, 22, 25). Lastly, 4) alteration of crayfish spatial behavior may affect as well symbiont fitness by
modulating transmission. Worm reproduction is increased at certain restricted sites on a crayfish, high worm densities; therefore, decrease the fitness of worms not inhabiting such good sites (Skelton et al. unpublished). Because branchiobdellidans are only transmitted by direct host contact (21), spatial movement which increases such contact would benefit worms on a crayfish with high branchiobdellidan densities.

We used the crayfish-branchiobdellidan system to examine symbiont effects on SMA under three distinct motivational scenarios: no symbionts, beneficial symbiont densities, and parasitic symbiont densities. We conducted a baseline study of crayfish with a range of naturally occurring symbiont densities that span beneficial effects at moderate values, and negative fitness effects at high values (21). We then verified baseline results by experimentally manipulating symbiont density. We show that the presence of symbionts qualitatively changes magnetic behavioral response to magnetic Earth-strength fields. Moreover, high densities of symbionts were shown to quantitatively suppress magnetically structured behavior. Our results indicate complex host-symbiont interactions modulate magnetic behavior and that the two cannot be considered independently.

Results

In our baseline study, with un-manipulated symbiont densities, crayfish aligned significantly along the NNE-SSW simulated magnetic axis (Fig. 1A). In contrast, no overall topographic alignment was detected (see Fig. S1A). Moreover, symbiont densities explained much of the variation in crayfish SMA responses. At moderate densities (<5 worms) SMA was highly significant (Fig. 1B) and topographic alignment was randomly distributed (Fig. S1B). The reverse was true of crayfish with high worm densities (≥ 5 worms) in which SMA was non-significant and topographic alignment perpendicular to the topographic north-south axis was significant (Fig. 1B and Fig. S1B). Therefore, high worm densities did not disrupt
alternative (non-magnetic) cues, but rather changed the relative utility or salience of magnetic cues.

**Figure 1: Magnetic alignment of crayfish.**

We tested magnetic alignment behavior of crayfish in a baseline assay without prior manipulation of ectosymbiontic worm densities (a). We then split the distribution of crayfish alignments based on symbiont densities above (high) and below (moderate) the median value of 5 worms (b). We also tested magnetic alignment behavior of crayfish with manipulated worm densities at three treatment levels; no worms, moderate and high worm density (c). Baseline test demonstrated a significant magnetic alignment of crayfish to the magnetic NE/SW axis. Splitting crayfish by worm density revealed that those with moderate worm densities were very significantly aligned, whereas those with high worm densities were not magnetically aligned. The experiment using three different ectosymbiont densities
showed significant bi-axial alignment without worms (no worms), axial alignment in the moderate worm density and no magnetic orientation in the high worm density. Significance of alignments was tested by Rayleigh-test; p-value (p), sample size and the mean vector length (r) are given for each distribution. We compared distributions using the Watson U²-test; test statistic (U²) and p-values are shown above the arrows indicating the compared distributions. All p-values with an asterisk are alpha corrected, when tested for axial and bi-axial alignment. We calculated a 95% around the mean direction confidence interval in case of significance.

Our symbiont manipulation experiment (with zero, moderate (6) and high (12) symbiont densities) confirmed our baseline observations that high symbiont densities caused a shift from SMA to a response to topographic cues. Again, crayfish with moderate densities of symbionts showed a strong magnetic alignment along the NE/SW axis and no significant topographic alignment. Also, alignments of crayfish with high symbiont densities were randomly distributed with respect to the magnetic field but significantly aligned to topographic cues and significantly different from the moderate symbiont density treatment (Fig. 1C and see Fig. S1C). Furthermore, circular standard deviation increased with symbiont density in both experiment, showing that increasing symbiont densities cause increased variability in host magnetic response (Fig. 2, see Fig. S2 for topographic relationship).
Figure 2: Variability in magnetic response as a function of symbiont density.

Comparison of the relationship between variability of crayfish magnetic orientation (circular standard deviation) and the number of symbionts during the baseline study with natural symbiont density (red circles) and experimentally manipulated symbiont densities (grey circles). Variability in magnetic orientation increased with increasing symbiont density in the baseline and manipulated experiments. Variability in baseline data was calculated as the moving circular standard deviation of orientation direction across a moving window of twelve individuals sorted by increasing observed natural symbiont density. Mean number of symbionts is the average number of *C. ingens* observed on the moving window of twelve individuals. Variability for manipulated symbiont density is shown as the circular standard deviation for each treatment level. Error bars show 90% confidence interval based on bootstrapped values from 10,000 resamplings without replacement. Treatment levels were adjusted to reflect symbiont attrition prior to testing. The no-worm treatment is not shown; this condition was not observed during the un-manipulated baseline study and caused a shift
to a different magnetic response (bi-axial), which would be misrepresented with an axial standard deviation.

In the absence of symbiotic worms, crayfish changed their magnetic spatial behavior; they aligned significantly bi-axial along the anti-cardinal magnetic directions (i.e. NE, SE, NW, SW) (Fig. 1B). The no-worm treatment and the moderate density treatment showed no topographic alignment (see Fig. S1B). The observed magnetic directional preferences did not include the direction of the animals’ native stream, nor the land-water axis in the confidence intervals, therefore suggesting an innate SMA behavior.

Also, the presence of branchiobdellidans affected host locomotor behavior by increasing host activity in the zero worm treatment, however, there was no difference between moderate and high worm treatments (Fig. 3, Two-way ANOVA, $F = 9.20, p < 0.001$).
Figure 3: Activity analysis.

Crayfish with worms applied showed significantly lower activity than crayfish without any ectosymbionts (in the worm-density manipulation experiment).

Discussion

In the present study we show, for the first time, that crayfish detect and respond to the Earth’s magnetic field. In baseline experiments, with unmanipulated ectosymbiotic worm densities crayfish align axially along the NNE/SSW axis. Such magnetic alignment behavior may be associated with a fundamental role of the Earth’s magnetic field in general movement behavior (1, 9-11, 28). For instance, the magnetic field has been suggested to function as a
rangefinder, which can help estimating distances and serves as a stable reference (1, 11, 14). The Earth’s magnetic field may also provide a magnetic grid which could be involved in everyday spatial behavior by constituting an important input in a ‘mental map’ structuring the animal’s environment and placing landmarks into register (1, 9-11, 28). Axial SMA, therefore, could be seen as a behavior to fall back on the reference direction in order to update the ‘mental map’ of the surrounding and assess potential changes in the environment (9).

The baseline SMA behavior found in crayfish, along the NNE/SSW axis, closely reassembles SMA responses in other animals. For example, resting and grazing cattle, roe deer and red deer show a remarkably similar orientation (29). Furthermore, carps, dogs, foxes and landing water birds show similar, presumably magnetic, preferences as well (14, 30-32). In general, data suggests that more stationary behavior leads to more pronounced axial SMA responses in animals (33).

However, in our experiment, we saw a switch to a different magnetic alignment behavior after removing all symbionts from the crayfish. Instead of aligning axially, animals magnetically aligned biaxially (quadramodally) on the anticardinal directions. In contrast to axial SMA, bi-axial SMA is mostly known from insects (for one exception see (34)), which tend to align on the cardinal magnetic compass directions when untrained. The examples include resting behavior in American cockroaches (35), but also magnetic movement preferences in adult fruit flies (36). Anticardinal bi-axial magnetic alignment preferences, as shown in our study after removal of all annelid ectosymbionts, has previously been observed in fruit fly larvae moving on a test plate (37). To the best of our knowledge the herein presented switch between two different SMA behaviors in a single species has never been demonstrated before.
We found consistent weakening of SMA with increasingly high symbiont densities in our baseline and symbiont manipulation experiments. One explanation for density-dependent effects of branchiobdellidan on SMA is that high densities merely provide distracting tactile stimuli that alter underlying behavioral motivation from responses to spatial cues, to grooming (e.g. (7)). However, this interpretation is inconsistent with our experimental results because a motivational change from spatial behavior to grooming would reduce activity, as crayfish remain stationary while grooming. In contrast we found no difference in overall activity between crayfish with moderate and high symbiont densities. Moreover, the observed changes in activity cannot be explained by the observed differences in magnetic alignment among symbiont treatments because high and medium symbiont density had significantly different alignments, but did not differ in activity. Instead, crayfish seem to switch to a different spatial strategy and respond to a topographic cue, which might indicate a general change of the spatial movement strategies used by the crayfish, either because the magnetic information is no longer available or its salience has changed.

Therefore, we suggest that density-dependent effects of branchiobdellidans on host and symbiont fitness may select for changes in spatial behavior that modulate symbiont density through transmission. Crayfish fitness is maximized at moderate worm densities (25), and strong intra-symbiont competition at high densities may decrease symbiont fitness. Because transmission of branchiobdellidans occurs mostly during direct physical contact among hosts (21), increased movement and altered spatial behavior can directly influence transmission rate by increasing host contact. We speculate that crayfish with no symbionts may show increased activity to seek contact with infected hosts and obtain beneficial symbionts. Furthermore, the bi-axial alignment in contrast to the axial baseline alignment might indicate a changed turning behavior, which potentially reflects increased exploratory behavior. The observed change in salience from magnetic to non-magnetic cues at higher
worm densities might be a result of host manipulation by the symbiont leading to increased transmission rates. At moderate densities, which are beneficial for symbiont and host, crayfish tend to remain more aligned along the NNE-SSW axis; this alignment behavior has been associated with resting behavior in other animals, as discussed above (29). One could speculate that by interfering with the magnetic behavior of the host, the crayfish switches to an alternative spatial strategy which might increase contact rate as well.

A growing body of evidence showing the magnetic field’s role in local movements and alignment behavior raises exciting new potentials for magnetic sensing across a diverse range of taxa (1). Symbioses are ubiquitous in nature and cause proximate changes in motivation and in sensory and motor integration. Given the importance of magnetic information in organizing spatial perception (38) and the ubiquity of symbiont influences on animal behavior, understanding how symbionts influence host magnetic responses is critical for understanding animal movement under natural conditions. Furthermore, a comprehensive understanding of the relationships between symbionts will not only help to account for variability often associated with studies of magnetoreception, but will provide novel insights into the evolutionary history underlying sensory systems and symbiotic interactions. Also, magnetoreception studies could benefit from an understanding of how previously overlooked biological interactions can alter magnetic responses and therefore might help to reduce variability of results concerning magnetic orientation. To the best of our knowledge, this is the first study to demonstrate that symbionts can alter their host’s behavioral response to Earth’s magnetic fields, an effect that could be easily overseen in un-manipulated field studies. Our findings go beyond previous work showing that symbiosis involves complex interactions between the host and symbiont with possible fitness consequences for both taxa, suggesting that these interactions involve a fundamental reorganization of the sensory input that organizes spatial behavior.
Material and Methods

Details of experimental animals

For all experiments crayfish (*Cambarus sciotensis*) were collected from Sinking Creek (Virginia, USA) (37.10°, -80.48°) one day prior to testing, held overnight at room temperature and brought to the testing facility (Behavioral Testing Facility, Virginia Tech, Virginia, USA) on the day of testing. The sampled stretch of the creek has a flow direction along the NW-SE axis (310°/130°), which results in a 40°/220° land-water axis (‘Y-axis’). Those directions were measured as alternative explanations for a magnetic body alignment, other than a spontaneous preference. The carapace length, mass and sex of all animals were determined prior to the experiments. The mean carapace length for the baseline test was 33.6 ± 3.1 mm and the mass was 14.2 ± 4.6 g. The mean carapace length for the worm manipulation experiment was 31 ± 3 mm and the mass 11.2 ± 4 g.

Procedure details

The animals were brought singly into the indoor testing rig and placed in radially symmetrical individual chambers (Fig. S3) in the center of a pair of cube-surface coils. The animals were transported in an opaque black container (13 cm *13 cm); the container was closed with a black opaque lid to prevent any visual cues while carrying the animal. Also, while carrying the animals the container was turned at least ten times to eliminate possible path integration cues. Reasons for exclusions of animals from the experiment were thunderstorms/rain events during testing and the death of animals in testing. After each test it was confirmed that each animal was alive; in three incidents the crayfish died in the experimental chamber and were therefore excluded from further analysis. A chamber outside
the Faraday cage filled with water, in the same way as the experimental chambers, was used as a reference for the water temperature.

For the baseline test air temperature was $24.6 \pm 2.2^\circ C$, and water temperature was $21.6 \pm 1.9^\circ C$ during testing. In the worm manipulation experiments air temperature was $19.9 \pm 2.5^\circ C$ and water temperature was $12.3 \pm 1.4^\circ C$.

**Magnetic field manipulations**

The magnetic fields were produced by double wrapped Rubens coils (39), producing a total field intensity of $50.99 \pm 0.37 \mu T$ and inclination angle of $64.5 \pm 0.7^\circ$, for all four fields, which reassembles an earth-strength magnetic field. Coils were used to rotate earth-strength magnetic fields into one of four cardinal alignments during each trial to uncouple magnetic field directions from all other fixed spatial references and allow us to separate magnetic and non-magnetic topographic responses (see Fig. S4). All experiments were conducted in a grounded Faraday cage. All field alignment changes were conducted from outside the Faraday cage without disturbing the animals.

**Video analysis**

A camera connected to a computer underneath the arena recorded the animals. From the recordings for each field treatment, one hour (out of two hours) was used in the case of baseline experiments and 30 min (out of one hour) in case of worm-densities experiments, deleting the first and last 30 min and 15 min, respectively. The videos were recorded in Virtual Dub, converted to one frame per second, and then tracked and analyzed using a custom-programmed tracking software ‘Alignment v4’ (programmed by co-author Rachel Muheim in Matlab R2012a, The Mathworks Inc.). The tracking software starts tracking an animal when the first movement is detected and records the axial body alignment (i.e. does
not distinguish between head and tail). The software also calculates an activity index, which is a measure for the movement of the animals, because it also includes angular movement it was not transformed into a distance measurement. For the activity analysis (comparing treatments in worm manipulation experiment) all 4 hours of videos were used.

**Standard circular statistic details and activity analysis**

The vector sum, combining the different field alignments, was calculated relative to the topographic and magnetic north. Using preliminary data as a reference, the expected orientation was an axial alignment response, however, the response to the worm treatments was unknown. Therefore we also tested for bi-axial alignment, if the distribution failed to be significantly axial; a Bonferroni correction was used to correct for multiple hypothesis testing in those cases. All circular statistics were conducted in Oriana 4 (40). Because bi-axial (quadramodal) orientation is not a standard function in Oriana 4, it was calculated by quadrupling the angles and then reducing the values to modulo 360° and testing for a unimodal orientation in Oriana 4. For the remaining analysis the data were treated as ‘axial’ in Oriana. Activity between treatments was compared using an ANOVA with day as a blocking factor, comparing each treatment using the Tukey method.

**Acknowledgments**

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10. Landler L, Painter MS, Youmans PW, Hopkins WA, & Phillips JB (under review) Spontaneous magnetic alignment by yearling snapping turtles: rapid imprinting-like association of radio frequency dependent pattern of magnetic input with novel surroundings. *PLOS ONE.*


Supplementary Figures

Figure S1: Topographic alignment of crayfish.

a) The overall baseline test showed no significant alignment relative to the topographic north. b) Crayfish with high worm density (≥5 worms) showed significant topographic alignment; moderate worm density (<5 worms) crayfish were randomly aligned relative to topographic north. c) The experiment using three different ectosymbiont densities showed non-significant topographic alignment in the no worm treatment and in the moderate worm density (m) treatment. However, high worm density led to a significant topographic alignment. All data are shown with axial data, no distinction between head and tail direction.
was made. Significance of alignments was tested by Rayleigh-test; p-value (p), sample size and the mean vector length (r) are given for each distribution. We compared distributions using the Watson $U^2$-test; test statistic ($U^2$) and p-values are shown above the arrows indicating the compared distributions. All p-values with an asterisk are alpha corrected, when tested for axial and bi-axial alignment. We calculated a 95% around the mean direction confidence interval in case of significance.

Figure S2: Topographic baseline response vs. worm manipulation experiment.

Comparison of the relationship between variability of crayfish topographic alignment (circular standard deviation) and the number of symbionts during the baseline study with natural symbiont density (red circles) and experimentally manipulated symbiont densities (grey circles). Variability in topographic orientation slightly decreased with increasing symbiont density.
diversity in the baseline and manipulated experiments. Variability in baseline data calculated as the moving circular standard deviation of alignment direction across a moving window of twelve individuals sorted by observed natural symbiont density. Mean number of symbionts is the average number of *C. ingens* observed on the moving window of twelve individuals. Variability for manipulated symbiont density is shown as the circular standard deviation for each treatment level. Error bars show 90% confidence interval base on bootstrapped values from 10,000 resamplings without replacement. Treatment levels were adjusted to reflect symbiont attrition prior to testing. The no-worm treatment is not shown; this condition was not observed during the un-manipulated baseline study and caused a shift to a different magnetic response (bi-axial), which would be misrepresented with an axial standard deviation.

**Figure S3: Experimental chamber.**

Crayfish were tested in visually symmetrical chambers, each one covered with a diffuser. Beneath each chamber a plastic mesh reflected the light and therefore prevented the animals from seeing the camera underneath.
Figure S4: Methods used to de-couple magnetic and topographic crayfish alignment.

a) Crayfish were tested singly in visually symmetrical chambers. Magnetic coils were used to alter the direction of simulated earth-strength magnetic north (mN) to all four cardinal topographic directions in randomized sequence. Shown is a rotation of mN from topographic north (left) to topographic east (right). b) Customized tracking software recorded the body axis orientation of crayfish (red dashed lines) during each trial at (1 s) intervals. For each crayfish under each magnetic field direction, the vector sum of all recorded orientations throughout the observation period were combined to generate a mean orientation vector. Shown are the mean orientation vectors when mN and tN are matched (black double-headed arrow) and when mN is rotated 90° to topographic east (blue double-headed arrow). c) Mean orientation vectors for all for magnetic field directions, for each crayfish, were added and the resultant mean direction was taken as the axial mean bearing. Mean vectors were
added as vectors relative to mN and vectors relative to tN, resulting in two different distributions, one showing the orientation in relation to the manipulated earth-strength magnetic north (left) and the other one showing the responses in relation to the unchanged topographic north (right).
5. The mysterious topographic bias: Magnetic effects on chicken embryo alignment not replicable?

Lukas Landler, Michael S. Painter, Paul B. Siegel and John B. Phillips

Introduction

A variety of animals exhibit magnetic alignment behavior, which implies, at least in certain situations, positioning of their body axis relative to the Earth’s magnetic field (Begall et al. 2013). Such behavior does not require training of the animals; it is a spontaneous magnetic response, whose function, if there is any, is still not understood. Although most magnetic alignment studies focused on adult animals, spontaneous magnetic responses in early life stages could be of particular importance, because of its potentials to study the ontogeny of a magnetoreceptor.

Saali & Juutilainen (1988) reported that earth-strength magnetic field changes can influence the alignment of the body axis even of the developing chicken embryo. Eggs incubated for approximately 72 hours were opened and the embryo body axes were recorded. A natural magnetic field data set was compared to an experimentally manipulated magnetic field data set (the magnetic field was altered 90° using Helmholtz coils). The two treatments led to differences in the embryo alignment distributions. It is important to note, however that only the altered magnetic field showed a significant orientation, the natural magnetic field
was randomly distributed, when using standard circular statistics (Rayleigh tests, (Batschelet 1981)). Because, both distributions were significantly different, suggested some magnetic effect on the body axis alignment of chicken embryos. Previously, Veneziano (1964), using bar magnets and 48 hours of incubation, also observed magnetic effects on the alignment of chicken embryos. The magnetic fields produced, however, were quite variable, between 3100 \( \mu T \) and 110 \( \mu T \); this is about 52 times and 2 times the Earth’s magnetic field strength, respectively. At those early embryonic stages the chicken ontogeny most sensory organs are not yet developed, and mechanisms underlying the formation of magnetoreceptors are unknown. A functional magnetoreceptor at this developmental stage could help studying the underlying magnetoreception mechanism and understanding the development of the magnetoreceptor itself. Therefore, we experimentally tested the hypothesis that chicken embryos align their body axis in relation to the Earth magnetic field in early stages of their development. Magnetic manipulation methods used allowed us to distinguish clearly between a response relative to the Earth’s magnetic field and a response to any other, non-magnetic cue.

**Methods**

The eggs used were from a flock of White Leghorns chicken selected for low antibody response to sheep red blood cells (Zhao et al. 2012) maintained at Virginia Tech. The eggs were put into an egg holder and glued to the bottom of the egg holder. Thirty eggs were tested simultaneously in each round. Each egg was turned at least 20 times (10 times counter-clockwise and 10 times clockwise) in order to avoid any preexisting directional bias. From test series 3 onwards the egg holder was turned 5 times inside the coil before the egg holder was put down pointing towards the right experimental direction. This was done to further reduce directional biases. Measurements of the axis of the embryo were made after
approximately 72 hours of development. Used were four different magnetic field conditions, which varied slightly between the experimental studies (magnetic north to topographic N, E, S, W, respectively). This experimental design enabled us to switch the magnetic field to all four cardinal directions and to distinguish between a topographic response (not changed by the change of the magnetic field direction) and a magnetic response (changing with change of the magnetic field) (Muheim et al. 2006). Methodological details for each test series are given in table 1.

**Incubation**

Two different types of incubators used throughout the experiment were a Styrofoam incubator and whole room incubation. The Styrofoam incubator was connected to a chiller, which pumped heated water through the incubator. In the whole-room incubation an oil filled heater in the experimental room heated the whole room to the incubation temperature (approximately 39°C).

**Vibration dampening**

For some test series the water lines to the Styrofoam incubator were run through buckets filled with cement and hammer arresters in order to reduce potential vibrations coming through the lines (for details see table 1). In later test series a sandbox was placed under the testing rig in order reduce potential vibration coming into the room through the floor.

**Magnetic coil set-up**

For most of the test series a pair of double wrapped 4 element Merritt et al. (1983) coils were used to produce a magnetic field almost identical to the local magnetic field
strength (see table 1 for values). For the last test series, in addition, a 3 element Merritt’s coil
canceled the Earth’s magnetic field and the original coils were used to re-create the magnetic
field. A square Helmholtz coil (Kirschvink 1992) was used to cancel the vertical magnetic
field component. All magnetic coils used were powered throughout all experiments; magnetic
fields were changed by reversing the polarity in one of the wraps in the double wrapped coils.

Statistics

Standard circular statistic was used to analyze the data (Batschelet 1981). To test for
significant unimodal orientation we used the Rayleigh-test. In test series without significant
unimodal orientation we also tested for bimodal orientation by doubling the angles and
reducing the results to modulo 360° (Batschelet 1981). The distribution (unimodal versus
bimodal) with the larger r-value (mean vector length) was then used for the test series. The
distributions were calculated relative to the topographic (unchanging) north (tN) and towards
the, for every test changing, magnetic north (mN).

Results

In none of the nine test series did the magnetic distribution show a stronger
orientation than the topographic distribution (figure 1). In three test series the magnetic
distribution almost reached significance, however, this happened because of an unequal
sample size per magnetic test field (this means that the two distributions, relative to tN and
mN, appear more similar). All, but one, topographic distributions reached significance either
unimodally or bimodally. The nonsignificant topographic distribution had a smaller sample
size in comparison to the other test series. Topographic orientation was overall approximately
aligned towards northeast, with only slight variations among test series.
Discussion

Studying the youngest ever-reported magnetic alignment behavior in animals would have allowed us to start asking questions about the development of magnetoreceptors. However, after many attempts, we failed to replicate the results by Saali & Juutilainen (1988). In our series of experiments an unknown secondary factor most strongly influenced the alignment of the chicken embryos. Furthermore, the increase in field strength (test series 9) did not improve the magnetic response, and the topographic bias stayed as strong as in the other test series. After all the measures that were taken to reduce potential directional sources of vibration, it is unlikely that vibrations coming from other operations in the laboratory were the cause of the topographic bias.

Furthermore the experimental procedure did not introduce any directionality; several rounds of turning should have equalized any pre-existing biases. In conclusion, although the embryos in our study did not show any magnetic response, they did orient with respect to an unknown cue, which was present throughout all experiments. Our results show that experimental designs used to study magnetic orientation need to decouple the magnetic component of orientation from any topographic component. It is conceivable that the magnetic effect reported by Saali & Juutilainen (1988) was the result of a topographic bias, and may have been introduced by switching on the magnetic coils.

Acknowledgement

We are grateful to Adrian Castaneda, Danneca Mundle, Katie Broadway for their help with the presented project.
Literature


Veneziano, P. P. 1964. The effect of low intensity magnetostatic fields on the growth and orientation of the early embryo of *Gallus domesticus* Evanston, III.


Table 1: Methodological details for each of the test series.

<table>
<thead>
<tr>
<th>Test series</th>
<th>Light/Dark</th>
<th>MF strength</th>
<th>incubator</th>
<th>vibration dampening</th>
<th>measures for RF reduction</th>
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</tr>
<tr>
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<td>none</td>
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<tr>
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<td>Styrofoam</td>
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<td>none</td>
</tr>
<tr>
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<td>none</td>
</tr>
<tr>
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<td>39 μT (+/- 2)</td>
<td>Styrofoam</td>
<td>1 cement bucket, 1 hammer arrester</td>
<td>RF filters</td>
</tr>
<tr>
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<td>39 μT (+/- 2)</td>
<td>Styrofoam</td>
<td>2 cement bucket, 2 hammer arrester</td>
<td>RF filters</td>
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<tr>
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<td>none</td>
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</tr>
<tr>
<td>8</td>
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<td>39 μT (+/- 2)</td>
<td>whole room sandbox</td>
<td>RF filters, temperature probe removed because of potential RF emission</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>24/0</td>
<td>89 μT (horizontal component only)</td>
<td>whole room sandbox</td>
<td>RF filters, temperature probes shielded</td>
<td></td>
</tr>
</tbody>
</table>

MF = magnetic field
RF = radio frequency
Figure 1: Results of test series 1 to 9 (a-i).

The sample size ($n$), mean vector length ($r$), and $p$-value ($p$) are given for each distribution. A single headed arrow indicates a unimodal distribution; a double-headed arrow a bimodal distribution, the length of the arrow is proportional to the mean vector length. The outer circle
would be a vector length of $r = 1$, the inner dotted circle represents the vector length needed for $p = 0.05$. For each of the test series the distribution is shown relative to the, unchanged, topographic north and the magnetic north, which was changed for every test.
6. Global trends in woodpecker cavity orientation: latitudinal and continental effects suggest regional climate influence

Lukas Landler, Michelle A. Jusino, James Skelton, Jeffrey R. Walters


Abstract

Animal constructions represent an extension of the individual phenotype upon which selection may act to create discernable population level patterns. Here we explore global patterns in woodpecker cavity entrance orientation to infer underlying selective forces that shape cavity construction. We performed a comprehensive systematic meta-analysis of woodpecker cavity entrance orientation from 80 populations of 23 species of woodpeckers and other picids throughout the Northern Hemisphere. We show that woodpecker cavity entrance orientation is typically non-random, suggesting that selection acts on cavity entrance orientation. Populations occurring at higher latitudes preferred a more southerly orientation, indicating that temperature or something related thereto may be driving cavity entrance orientation. Differences between Eurasia and North America in orientation are consistent with this hypothesis. Taxonomic relationships were not a significant predictor of the strength of orientation and thus unrelated woodpecker populations respond similarly to regional
environmental drivers. Our results demonstrate latitudinal and continental patterns that strongly suggest regional climate as a selective force on cavity entrance orientation. Further work utilizing available long-term data sets throughout the world promises to uncover possible fitness consequences of cavity orientation on woodpeckers.

Introduction

A variety of animals act as architects by modifying their environment to create structures to provide food or shelter, or attract mates (Hansell 2005). Well-known examples of animal architecture include termite mounds (Korb & Linsenmair 1999), fish nests (Kawase et al. 2013), rodent burrows (Dawson et al. 1988) and bird nests (Hansell 2000). Construction of animal architecture has impacts that expand beyond the architects themselves because the built structures provide habitat and resources for a variety of other taxa (Coleman & Williams 2002). Therefore, animal architects can be considered to be ecosystem engineers (Jones et al. 1996) and the selective forces that act on architects and guide the structure and function of their constructions may have community-level implications (Wright & Jones 2004). For instance, in certain cases, woodpeckers create new niches through excavation in systems where naturally formed cavities are not abundant, thereby providing habitats for a variety of secondary cavity nesters (Aubry & Raley 2002, Blanc & Walters 2008, Saab et al. 2004). While there are numerous studies that investigate the structure and function of insect buildings or mammalian tunnel-systems and nests, bird architecture is much less understood (Hansell 2000).

The alignment of animal constructions can influence their function and provide evidence of the underlying selective forces and environmental cues that guide animal architecture. Animal buildings represent an extended phenotypic trait (Dawkins 1982),
meaning that selection acts on the structure and function of an animal’s buildings, as on other phenotypic traits of the animal. Selection on extended phenotypes can be expected to be strong if the individual invests considerable time and energy in their construction. The geographic alignment of a structure is one potentially important facet of animal construction upon which selection may act. Building alignment can be defined either as the direction of the longest axis of a structure or, in cases of open nests, as the direction towards the open area (e.g. when a nest is leaning against a tree, or a wall). Alignment can have effects on the microclimate of a structure via convection by wind and exposure to solar radiation (Hansell 2005). For instance, observed patterns of alignment in termite mounds may represent a trade-off between thermoregulation and gas exchange (Korb & Linsenmair 1999). Thus, selection may favor individuals who align their construction in directions that increase their fitness, creating non-random alignment patterns at the population level (Goodenough et al. 2008).

Woodpeckers all over the world construct and/or use tree cavities for nesting and roosting. In many cases cavity entrance directions show non-random orientation patterns in various woodpecker populations (Inouye et al. 1981, Locke & Conner 1983, Wan et al. 2008), suggesting that selection favors individuals within these populations that align their cavities to a particular direction. Selective cavity entrance orientation may be advantageous for woodpeckers in at least two ways: the effort required for excavation may be reduced and breeding success could be increased with certain alignments. Both mechanisms have been discussed previously (Conner 1975, Wiebe 2001), though previous studies have been largely limited to single localities or single woodpecker taxa. Case studies provide valuable insights to the taxon-specific and local habitat variables that may account for observed patterns in cavity entrance orientation, but leave one wanting a more general understanding of the importance of cavity entrance orientation to woodpecker biology. Cavity entrance orientation
is often recorded in the field and several long-term study sites of cavity-nesters exist around the world, providing an excellent basis for a synthetic analysis of the causes and consequences of non-random cavity entrance orientation in woodpeckers.

Our goal is to highlight the widespread ubiquity of non-random woodpecker cavity entrance orientation and stimulate future scientific inquiry into its causes and consequences. Here we survey the literature to address questions concerning the general importance of cavity entrance orientation to woodpecker biology. First we ask if non-random cavity entrance orientation is commonplace among woodpecker populations of multiple species and across a broad range of geographic locations. Second, we ask if there are geographical, ecological, or phylogenetic patterns in the direction of alignment that may suggest underlying causative mechanisms. We also provide recommendations to guide appropriate future studies of woodpecker cavity entrance orientation. A better understanding of cavity entrance orientation and its significance to the success of cavity excavators may be of great value to evolutionary studies of cavity-nesting communities and may inform conservation efforts by providing a better understanding the nest site requirements of these ecosystem engineers.

**Methods**

**Do woodpeckers have a preferred cavity entrance orientation direction?**

To determine if non-random cavity entrance orientation is a general phenomenon among woodpecker populations, we performed a re-analysis of published data. In a first step data were obtained from a systematic literature search using Google Scholar® and the English search terms “[common name] cavity orientation” and “[scientific name] cavity
orientation”. Google scholar® is a freely accessible and comprehensive global scientific search engine, which allows for an efficient and systematic literature search available to everyone. Using current species checklists for the 5 continents on which woodpeckers and other picids exist, we searched over 200 Picidae species names from North, Central, and South America, Eurasia, and Africa. In order to capture more representatives of European species, we also conducted searches for European species in German language, and likewise we conducted additional searches in Spanish in order to capture more Central and South American species. In addition to our systematic search we also opportunistically included papers that were mentioned and made available to us by other researchers.

From the studies found through our systematic and opportunistic search, we include here only those that provided sufficient data in the original manuscript for re-analysis using standardized methods. We excluded studies in which the species associated with cavities could not be determined unambiguously, and studies examining artificial cavities such as nest boxes or human-made tree-holes. In some cases where tabular data were not provided in the original manuscript, data were inferred visually from graphical representations. We also included studies that reported orientation statistics but did not provide data in tabular or graphical form, if the results of appropriate analyses were reported in the original manuscript. Using these criteria we found 46 studies from North America, 30 studies from Europe, and 4 studies from Asia from 53 publications (some publications contain studies of more than one species) to include in our analyses, representing 1-12 studies per each of 23 Picidae species (Table 1). Because Europe and Asia represent a single landmass and the separation is founded on cultural rather than scientific reasons, and we recovered relatively few Asian studies, European and Asian studies were combined as ‘Eurasia’.
To determine the prevalence, geographic, and phylogenetic patterns of cavity orientation in Picidae populations, we re-analyzed all available data sets using a standardized method to allow for cross-study comparisons. Data from each report were analyzed using the Rayleigh-test (Batschelet 1981) to test for a significant deviation from a random distribution (alpha = 0.05) and strength of orientation. The strength of the deviation from random is represented by r, which ranges from 0 to 1, 0 indicating a perfectly random distribution and 1 representing perfect alignment. The Rayleigh-test is the most common statistical test used to analyze circular data for significant unimodal orientation and it is a standard statistical function in all circular statistic software packages (e.g. Oriana; Kovach 2011, or the R-packages ‘circular’; Lund & Agostinelli 2011, and ‘CircStat’; Lund & Agostinelli 2009). In this meta-analysis, we used the circular statistic software package Oriana 4.0 for all circular re-analyses.

Because the studies surveyed varied greatly in total sample size (n = 3 to 437), and sample size has a large influence on statistical power, we correlated the binary response variable of significance (1) versus non-significant (0) results with study sample size to estimate the degree to which published non-significant findings were due to under-sampling. This binary response analysis allowed us to make an estimate of sufficient sample sizes for determining non-random cavity orientations in woodpecker populations. We used a generalized linear model (GLM; function glm, R base package v 2.15.1) to estimate the proportion of studies that found significance as a function of sample size, assuming a binomial error distribution.
Global patterns of woodpecker cavity orientation

Using the published datasets described above, we investigated global patterns in woodpecker population cavity orientation to determine if orientation varies predictably with geographic location by examining the effect of latitude and continent (North America and Eurasia) on the mean direction of picid cavity orientation. For this analysis, we only included studies that provided evidence of significant non-random orientation according to our re-analyses (alpha = 0.05, n = 31). We used generalized linear models (GLM; function glm, R base package v 2.15.1, family = gaussian) to model the singular effects of latitude and continent, and the interaction of latitude and continent, on deviation of the population mean vector from north. Angular deviation from north was used to linearize circular data, i.e. an orientation towards south would have the maximum value of 180, a northward orientation the minimum value 0. However, orientation towards east or west would both be 90. Model fit was assessed visually and non-significant predictors and interactions were sequentially dropped during model selection.

Results

Do woodpeckers have a preferred cavity entrance orientation direction?

Of 80 studies, 39% (31 studies) showed a significant cavity entrance orientation preference (Table 1). Sixteen of the 23 species considered (70%) had a significant orientation in at least one study. The proportion of studies in which significant results were found increased significantly with sample size (z = 3.636, p = 0.0003, Figure 1). By using a GLM approach, we estimated that more than half of the studies with a sample size of at least 100 showed non-random cavity entrance orientation (Figure 1). All studies for the 7 species for
which we found no evidence of non-random cavity entrance orientation had relatively small sample sizes (max = 47, mean = 26 +/- 14 SD). Those seven species were: White-headed Woodpecker (*Picoides albolarvatus*), Syrian woodpecker (*Dendrocopos syriacus*), Red-headed Woodpecker (*Melanerpes erythrocephalus*), Red-bellied Woodpecker, (*Melanerpes carolinus*), Red-breasted Sapsucker (*Sphyrapicus ruber*), Lesser Spotted Woodpecker (*Dendrocopos minor*), and Downy Woodpecker (*Picoides pubescens*).

![Figure 1: Proportion of significant studies in relation to sample size.](image)

The proportion of studies in which a significantly non-random cavity entrance orientation was found increased rapidly with increasing sample size ($z = 3.636, p = 0.0003$). Solid black line shows GLM model fit, +/- standard error (dotted lines). Data are represented by grey "|" symbols.

In order to compare the mean strength of orientation (the mean vector length ‘r’, used in the Rayleigh’s test) among genera of Picidae we used a one-way ANOVA. We only
included genera for which sample size was greater than four studies, thus excluding Picus (n = 2). There was no detectable difference in strength of alignment across genera (one-way ANOVA, F=1.816, p = 0.12, df = 5).

Global patterns of woodpecker cavity orientation

There was a highly significant global relationship between population latitude and the mean deviation from north in cavity entrance orientation (t = 3.746, p = 0.0008; Figure 2). Interestingly, the mean deviations differ significantly between North America and Eurasia, showing a more southerly alignment in North America compared to Eurasia in relation to latitude (t = 3.074, p = 0.005; Figure 2).
Figure 2: Shift in deviation from North with latitude.

Relationship between latitude of study site and angular deviation of study population mean vector from North for all studies from which we could extract a mean orientation vector (n = 31). Deviation from North increased with latitude (t = 3.746, p = 0.0008), and was greater in North American studies (black lines ± 95% CI and closed symbols; t = 3.074, p = 0.005) than Eurasian studies (gray lines ± 95% CI and open symbols) indicating a more southerly alignment in studies conducted at high latitudes and on the North American continent.
Discussion

Do woodpeckers have a preferred cavity entrance orientation direction?

Though woodpecker cavity entrance orientations are often recorded, many reports include no or inappropriate statistical analyses, and instead provide only qualitative descriptions of the observed cavity orientation data. In other cases, proper analyses reveal non-random alignments but interpretation of the findings is restricted to anecdotes. Some have argued for random cavity entrance orientation in birds (Blume 1961). Others have recognized non-random cavity entrance orientations, but dismissed them as “largely a statistical phenomenon” (Kerpez & Smith 1990), arguing that because high amounts of dispersion typical to cavity orientation data require large sample sizes to detect non-random patterns, a significantly non-random mean direction would not be biologically meaningful. Although large sample sizes do increase the power of an analysis to detect non-random patterns, we emphasize that they do not increase the likelihood of false positive results. Proper statistical tools and analytical methods for circular data provide robust tests for non-random patterns in orientation data (Batschelet 1981, Fisher 1995). Ecological and behavioral data are often plagued by high variance, but dismissing significant patterns as merely statistical phenomena may cause researchers to overlook potentially important natural processes such as natural selection on cavity entrance orientation.

The lack of a difference in the strength of orientation between genera suggests that cavity entrance orientation preferences are ubiquitous among woodpecker taxa and not limited to any particular subset of genera. Our results also suggest that the main reason for the lack of significant cavity entrance orientation in some studies is due to under sampling
and not due to a lack of orientation. We suspect that more rigorous sampling of populations of picid species will uncover more non-random patterns in cavity entrance orientation. Together, our results show that non-random cavity entrance orientation is commonplace among woodpecker populations and therefore likely to be an important facet of the biology of this group.

**Global patterns of woodpecker cavity orientation**

Considering the hypothesis that yearly mean temperatures influence cavity entrance orientation of woodpeckers (Wiebe 2001), one would expect northern populations to orient their cavity entrances more towards the south than southern populations, as more northern populations would benefit more from the warmer cavity temperatures provided by a southerly exposure. Indeed, our results overwhelmingly demonstrate the expected effect: cavity entrance orientations are highly significantly more southern oriented with increasing latitudes. Furthermore, we found that the orientation of North American populations were on average more southerly oriented than Eurasian populations of similar latitudes. If cavity temperatures are driving cavity orientation, such a difference is expected, as European climates are generally warmer than the North American climates of similar latitude due to warming influences such as the Gulf Stream. For instance, in January parts of Western Europe can be as much as 15°C to 20°C warmer than the same latitudes in North America (Seager et al. 2002). Therefore, these findings together provide a strong case for temperature as the major driver of global cavity entrance alignment direction in picids. Thus our results are congruent with other studies indicating population-level variation in bird constructions is related to thermal factors, such as those showing local temperature effects on the nest mass of Blue Tits (*Cyanistes caeruleus*) and Great Tits (*Parus major*) (Deeming et al. 2012).
While our work emphasizes that regional temperature shapes average population-level cavity orientation on a global scale, factors such as wind and microclimate may have effects at smaller, regional or local scales. Such effects could account for the frequently observed high degree of individual variation in cavity entrance orientation within many populations, such as those noted by Kerpez & Smith (1990). An interesting topic for future research could be to investigate different cavity entrance orientations at different time points during the breeding season, such as comparing alignments of first nests to those for second nesting attempts and any subsequent breeding attempts. For instance, Cactus Wrens *Campylorhynchus brunneicapillus* build closed nests facing away from the prevalent wind direction during the cool part of their breeding season (March and April) and towards the wind during the hotter portion of their breeding season (May and June; Ricklefs and Hainsworth 1969). In contrast to other bird species, woodpeckers and other picids have rather permanent constructions, and for certain species with longer excavation times, such as the Red-Cockaded Woodpecker (*Picoides borealis*) and the Black Woodpecker (*Dryocopus martius*), it may not be feasible to construct a new breeding cavity in order to change the orientation of a nest during the breeding season. Instead, woodpeckers that live in territories with multiple cavities could choose a different available cavity with a different cavity entrance orientation for a second breeding attempt than that used in their first attempt.

**Does cavity orientation affect Picid fitness?**

Our review uncovered a paucity of efforts to relate cavity construction to individual fitness. One study of Northern Flickers related cavity orientation to the fitness consequences of nesting site selection (Wiebe 2001), focusing on the relationship between cavity microclimate and hatching and fledging success. Cavity entrance orientation in the study population was indeed oriented towards south (210°), and Wiebe (2001) showed that
southerly-aligned cavities yielded warmer cavity temperatures than more northerly-aligned cavities. However, a relationship between cavity temperature and fledging success was not found, suggesting that something other than directional effects on fledgling success caused the observed southerly orientation. One potential alternative explanation is that excavation is facilitated in certain directions due to regional variation in the directional growth of decay fungi. Most decay fungi present within excavation sites are not initially noticeable with visual observation (Jusino et al. *in press*) and many excavation sites with decayed wood do not exhibit external signs of decay (Zahner et al. 2012); thus uneven decay within trees housing excavation sites would be difficult to visually assess in standing trees. Decay fungi are thought to facilitate cavity excavation (Cockle et al. 2012, Conner et al. 1976, Jackson & Jackson 2004, Jusino et al. *in press*, Witt 2010, Zahner et al. 2012), consequently a fitness advantage of cavity orientation could come from conserved energy during the excavation process rather than increased survival of offspring.

Furthermore, in this review we observed that data collected for woodpecker excavations often do not distinguish excavations based on their usage, such as roosting versus nesting or between cavity orientations on different tree species. However, at least the latter parameter could have important effects on cavity entrance orientation, as it was indicated in a study by Mazgajski (1998). Nonetheless, several efforts from researchers studying non-excavating species give promise that orientation and nesting success may be linked. In Black Kites (*Milvus migrans*), rainfall and prevailing winds appear to affect nest orientation. Nests which aligned to the east, which was also the preferred direction, were less exposed to rain and wind and were more successful (Viñuela & Sunyer 1992). Additionally, Cactus Wrens were found to have higher breeding success when their nests are aligned towards the predominant wind direction (southwest) in the late breeding season (Austin 1974).
We suspect that future comprehensive studies analyzing fitness effects of cavity entrance alignment direction will uncover the biological significance of alignment of woodpecker nest cavities and that this topic will provide a fruitful avenue of future research (Goodenough et al. 2008). Woodpeckers spend considerable amounts of energy during cavity excavation, thus this behavior likely is subject to strong selection. Surprisingly, cavity entrance orientation has not gotten the scientific attention that other variables in nest site selection and cavity construction have: for instance, a search on Google scholar® comparing the search term “woodpecker cavity orientation” with “woodpecker cavity height” revealed 3,410 and 10,200 hits, respectively (Google scholar® search on Oct 7 2014). We suggest two possible reasons for this imbalance of investigation. First, large sample sizes are often needed to detect fitness effects and in the case of cavity entrance orientation the actual benefit might be small. However, because orientation mechanisms are well developed in birds, choosing a certain direction for excavation might not require any substantial cost. Therefore, cavity orientation could very likely provide net fitness benefits, but these benefits might be difficult to detect with low sample sizes. Second, cavity orientation data are circular. Circular statistics remain a specialized field and the development of rigorous analytical tools for relating circular predictors to non-circular response variables, such as reproductive success, is a persistent and largely unresolved challenge. We are optimistic that these challenges will soon be met as more research is directed towards orientation studies, circular analytical tools become better developed and more available, and wide-spread fascination for the selective advantages of directional orientation unites specialists from the organismal biology and analytical realms.
Conclusions

This review is the first attempt to summarize woodpecker cavity entrance orientation data on a global scale and investigate the underlying factors governing orientation of cavities. Our reanalysis of published orientation data clearly shows that most woodpecker populations for which sufficient orientation data are available show significantly non-random cavity orientations. These results strongly suggest that selection acts on individual cavity entrance orientation, but the magnitude and precise nature of such selective forces remain unknown. Quantification and characterization of the effect of cavity entrance orientation on individual woodpecker fitness will provide fruitful future work to advance our understanding of woodpecker biology, the evolution of cavity excavation, and the interface between animal extended phenotypes and the natural environment. Furthermore, entire communities of secondary cavity users depend on the excavations of woodpeckers and different taxa may be differently affected by cavity entrance orientation. Therefore, selection on excavation orientation in woodpeckers may have effects that extend to entire communities of cavity inhabitants, and thus the selective forces that act directly on excavators may act indirectly to shape diversity in communities of organisms, which depend on woodpecker excavations. Cavity directionality is a standard measurement for field protocols, but rarely do researchers invest time for a thorough analysis of this cavity feature. However, long-term data sets, with sample sizes above 100 cavities, could be easily used to explore some of the questions we raised in this review, including the question of fitness consequences of cavity entrance orientation. Although we attempted to create an inclusive data set on a global scale, there is an apparent lack of searchable published accounts from Central and South America, Asia, Africa, and southern parts of Europe. We hope that this review initiates discussion, data collection in under-represented regions, and further comprehensive analyses of woodpecker
cavity entrance orientation in order to gain a better understanding of woodpecker excavation biology and the evolutionary interplay between environment and the extended phenotype.

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Pynnönen A. 1939. [Contributions to the knowledge of the biology of finnish woodpeckers].
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Table 1: Data summary of all publications that were included in the meta-analysis. Data are sorted by the mean latitude of species and by latitude of study sites.

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<td>USA, Idaho</td>
<td>Butcher et al. 2002</td>
<td>89</td>
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<td>Downy Woodpecker</td>
<td>Canada, Ontario</td>
<td>Lawrence 1967</td>
<td>11</td>
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<td>(Picoides pubescens)</td>
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<td>Bonferroni-corrected p-value</td>
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<td>Acorn Woodpecker (Melanerpes formicivorus)</td>
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<td>ENE</td>
<td>Hooge et al. 1999</td>
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<td>USA, New Mexico</td>
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