The Journal of Experimental Biology 209, 3882-3886
Published by The Company of Biologists 2006
doi:10.1242/jeb.02456

Laboratory behavioural assay of insect magnetoreception: magnetosensitivity of *Periplaneta americana*

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Accepted 24 July 2006

Summary

A relatively simple all-laboratory behavioural assay of insect magnetoreception has been developed. We found non-conditioned reactions of American cockroach to the periodical shifts of the geomagnetic field. The movement activity of animals individually placed into Petri dishes was scored as a number of body turns. Test groups were exposed to a 90-min interval with the horizontal component of the geomagnetic field periodically rotated by 60° back and forth with 5 min periodicity. The number of body turns was compared with the preceding and following intervals and with the corresponding interval of the control group kept in the natural field. We obtained a significant increase in activity when changes in field were applied. Interestingly, the period of increased activity did not coincide precisely with the 90 min stimulation interval. The onset of animal restlessness was delayed by tens of minutes and persisted correspondingly after the stimulation stopped. A respective evaluation criterion was suggested and verified.

Owing to its simplicity and minimal manipulation of the insects, together with low demands on the memory and motivation state of animals, the approach potentially may be used as a laboratory diagnostic tool indicating magnetoreception in insect neurophysiology research.

Key words: magnetoreception, insects, *Periplaneta americana*.

Introduction

In the last two decades, the phenomenon of magnetoreception has been convincingly demonstrated in a number of diverse animal species (Wiltschko and Wiltschko, 1995; Wiltschko and Wiltschko, 2005). Our contemporary knowledge of the phenomenon is based primarily on behavioural data. However, behavioural studies *per se* cannot, without subsequent neurophysiological analyses, elucidate transduction processes that occur at or below the cellular level (Johnsen and Lohmann, 2005). Recently, research on the remarkable animal sense for geomagnetic field faces the problem of identification of reception mechanisms including neural pathways processing the magnetic information. Such questions have not been answered satisfactorily in any animal.

Paradoxically however, one of the factors inhibiting progress in neurophysiological analysis of the magnetoreception may be the lack of appropriate behavioural laboratory assays on model organisms (Johnsen and Lohmann, 2005). In terms of organisms, a major part of current behavioural data has been obtained on vertebrates: birds, newts, turtles. Surprisingly, insects and other invertebrates are represented relatively sparsely.

Concerning the insect geomagnetic sense, no simple behavioural experimental paradigm has been transferred as a routine tool into neurophysiological laboratories comparable to research, e.g. on the molecular base of biorhythms (Sauman et al., 2005; Stoleru et al., 2004), smell (McGuire et al., 2005) or memory and learning processes (Pinter et al., 2005; McBride et al., 1999). Substantial analysis of insect magnetoreception processes, however, will not be possible without well linked behavioural and neurophysiological approaches. Apparently, the availability of suitable assays of insect magnetoreception is still rather limited.

In the last decades the most impressive series of experiments on insect magnetoreception was performed with honeybees (Kirschvink et al., 1997; Kirschvink and Kirschvink, 1991; Walker and Bitterman, 1989). The authors used a classical conditioning design, teaching bees to distinguish the presence of magnetic anomaly by means of a reward/punishment training paradigm.

An even more relevant species, *Drosophila*, was the object of another important conditioning experiment (Phillips and Sayeed, 1993). Fruit flies learned the magnetic position of the source of light that attracted them. In spite of the fact that other diverse experimental approaches were published (Acosta-Avalos et al., 2001; Banks and Srygley, 2003; Camlitepe et al., 2005; Etheredge et al., 1999; Perez et al., 1999; Srygley et al., 2006; Ugolini, 2006; Vácha and Soukopová, 2004; Zhang et

al., 2004) none has become a routine model for studies on neural substrate of the insect geomagnetic sense. We assume that either the strong binding to the open air environment or the complexity of laboratory tests and hence high degree of manipulation with living objects might be a significant reason. A more sophisticated assay also means a higher risk of a hidden factor that may be overseen when replicated.

We attempted to find a non-conditioned as much as possible robust and easily reproducible magnetoreception insect assay, with a minimum of manipulations and experimentalist's interventions.

In the 1960s a series of experiments was published (for a review, see Wiltschko and Wiltschko, 1995) reporting spontaneous magnetically aligned resting positions of termites, Diptera and other insects. We proceeded from the idea that unsteady magnetic field with the horizontal component periodically rotating back and forth may provoke more frequent position changes of resting insects compared to a natural steady field.

As a model organism the American cockroach *Periplaneta americana* L. was chosen; this is a classical species widely used in insect neurophysiology, however, having no recent evidence of magnetoreception skills.

Materials and methods

Animals

Cockroaches *Periplaneta americana* L. were kept in translucent plastic buckets with a wire mesh in the lid. Diet consisted of cat food pellets; a vessel with wet cotton wool was permanently present. The buckets were placed in containers in the rearing room and kept at a temperature of 26°C (±2°C) with a 12 h:12 h light:dark regime (from 06:00 h to 18:00 h). The bucket with cockroaches intended for the experiment was placed into a refrigerator (~4°C) for 30 min and the cold-immobilised animals were transferred individually into glass Petri dishes (diameter 15 cm, height 2.5 cm). Only adults (regardless of sex) were selected for the experiments.

Testing room

The design of the laboratory setup was described in detail elsewhere (Vácha and Soukopová, 2004). The testing room was on the third floor with regular office operations in the neighbourhood. The room was darkened and the same light:dark regime was set as in the rearing room. The temperature was kept between 20°C and 23°C. A hot air fan with a permanently running electromotor was suspended from the ceiling 2 m directly above the testing table.

During the test, the dishes with cockroaches were placed on a glass plate on top of the wooden table. A white paper ring (height 3 cm) encircled each dish so that the animals had mutually no visual contact. A round window (diameter 60 cm) cut into the desk under the plate allowed images of the animals' positions to be taken from below, using an Ikegami ICD 47 camera (Tokyo, Japan) located 1 m under the table. The space beneath the desk including the camera (except the lens) was covered with a black cloth. Depending on the number of animals per experiment, from three to nine Petri dishes (with one animal each) were ringed with a circular arena (diameter 56 cm, height 42 cm) having an opaque white inner surface. To diffuse the light, the area was covered with a lid of translucent Perspex having a sheet of white filter paper on its top. A frosted white light bulb (40 W, Phillips, soft tone) placed 50 cm above the lid illuminated the experimental space. Therefore, the cockroaches could see only the white lid, the white walls around them and the black cloth below. The arena rim was divided into 48 sectors and the centre of the arena was marked (visible only on the PC monitor) making it possible to determine the positions of the animals.

Magnetic conditions

The natural geomagnetic background in the laboratory was as follows: horizontal component 17.0 μT, inclination 69°; spatial variation in the region of the arena was <2% (measured by HMR 2300 magnetometer Honeywell, USA; EDIS software, Slovakia). Only the horizontal component was experimentally rotated by 60° CW by means of a horizontal four-element coil (size 2 m×2 m×2 m) (Merritt et al., 1983). The angle between the coil axis and the horizontal geomagnetic vector was 120°. The intensity of the artificial magnetic vector generated by the coil was identical to the natural horizontal component of the geomagnetic field. Feeding to the horizontal coil was changed from 0 A to 0.93 A at 5-min intervals (see below). The current upper limit was permanently set on the DC power supplier (DF1730SB, China), and change between the natural (0 A) and rotated (0.93 A) horizontal component of the field was executed manually by a turn of the voltage knob (lasting from 0.5 to 0.7 s). Both the frame-storing computer and the power supply were located in a separate room 10 m from the experimental lab. The experimental room was not equipped with a particular shielding system (Faraday cage) but the coil system was permanently grounded by means of the power supplier, which was switched on all the time.

Photic conditions

The white light bulb illuminated the arena through the lid diffusing the light so that its intensity on the bottom was 273 cd m^{-2} at the centre of the arena and 239 cd m^{-2} by the wall line (International Light IL700, SHD 033 probe, USA).

We decided to record the body turns activity of cockroaches during their minimal locomotion activity, at around noon. The aim was to minimize disturbing impacts of escape attempts, searching for food or partners, body cleaning etc., all of which may bring a noise into the activity data.

We scored the number of body displacements when the body axis slewed by more than 15° (2 sectors in the arena). The time schedule of the experiment is given in Fig. 1. The cold-immobilised animals were placed into Petri dishes at 16:00 h (±30 min). The dishes were placed on the glass plate and covered by the arena with the lid. At 10:00 h (±15 min) the following day, automatic computer recording and storing of the frames began and lasted till 14:30 h. The frequency of sampling

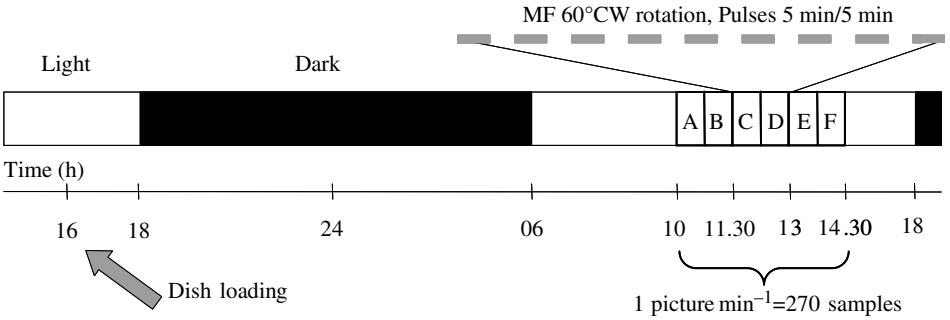

Fig. 1. Time schedule of the experiment. Animals were put into dishes at 16:00 h on the day before sampling. Sampling began at 10:00 h and consisted of pre-treatment periods A and B, magnetic treatment periods C and D and post-treatment periods E and F. Magnetic treatment was designed as nine pulses (5 min each) periodically shifting the horizontal vector of geomagnetic field (MF) by 60° clockwise (CW).

was 1 sample min^{-1} giving 270 frames for each animal. The whole period was divided into six 45 min intervals: the first two (A,B) prior to the magnetic stimulation, the middle two treatment intervals (C,D) when the field was rotated back and forth by 60°CW with a frequency of 1 per 5 min and the last two intervals (E,F) after the magnetic treatment. In addition to this experimental scheme we inserted control samples having the magnetic field natural all the time. Testing and control days alternated regularly.

Preliminary tests on 28 treated and 28 control animals showed differences in time dynamics between both groups (Fig. 2A). In the test group, increase in the number of body turns following the magnetic treatment is apparent. Remarkably however, the onset seemed to be delayed and higher activity persisted to, and peaked at, the following interval E although the field was steady again. No such effect was apparent in the control group. To capture this inertial after-effect we extended the treatment interval to period E. This way we obtained two intervals to compare: the extended, critical interval CDE and pre- and post-treatment intervals ABF, both with the same number of samples.

On the basis of the preliminary data, we set the contrast CDE/ABF as a main diagnostic indicator of positive magnetosensitive reaction. To prove this working hypothesis, in the next step, we set out to test a more extensive sample.

Analysis of preliminary results also showed the need to eliminate escapees: individuals trying to escape by surveying the walls and the lid of the dish for tens of minutes, unlike the majority of animals with lower movement activity that changed their body orientation only semi-occasionally. As a most acceptable criterion for filtering the escapees off, we set the number of 20 direct contacts of the head to the dish wall as the maximal tolerable escape activity. Individuals showing more than 20 head–wall contacts were discarded from the experiment. The body displacements accompanying contact with the wall or cleaning positions (body curved randomly) were not scored.

In consequent major series, we tested a total of 255 individuals, of which 59 were discarded for high escape activity (23%). The final experimental sample (with periodically shifting the middle interval) comprised 97 individuals whereas the controls (natural field) comprised 99 individuals.

Statistics

To compare the numbers of the body axis turns we used standard methods of non-parametric statistics (Mann–Whitney

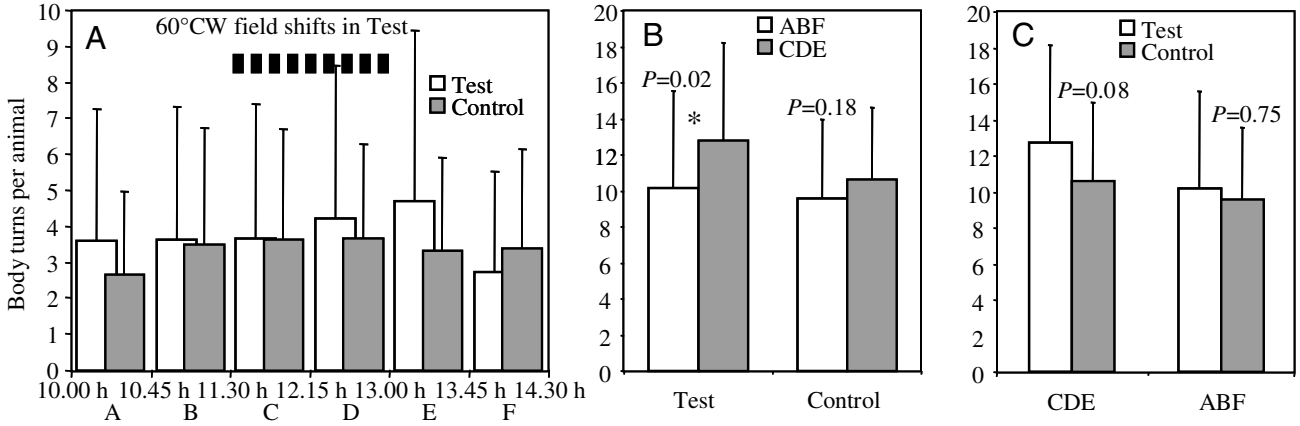

Fig. 2. Preliminary test series. Turning activity of animals in the natural field (Control) is shown as solid bars and in a field periodically rotated in periods C and D (Test) as open bars. (A) Unlike the control animals, activity increases in the test animals after the magnetic treatment is applied and persists to period E. (B) The contrast in the CDE *versus* ABF periods is significant for the Test group (Wilcoxon, N=28, P=0.02) but not for the Control group (Wilcoxon, N=28, P=0.18). (C) In the critical CDE period, activity in the Test group is higher then in the Control group yet not significant (Mann–Whitney, N=28/28, P=0.08).

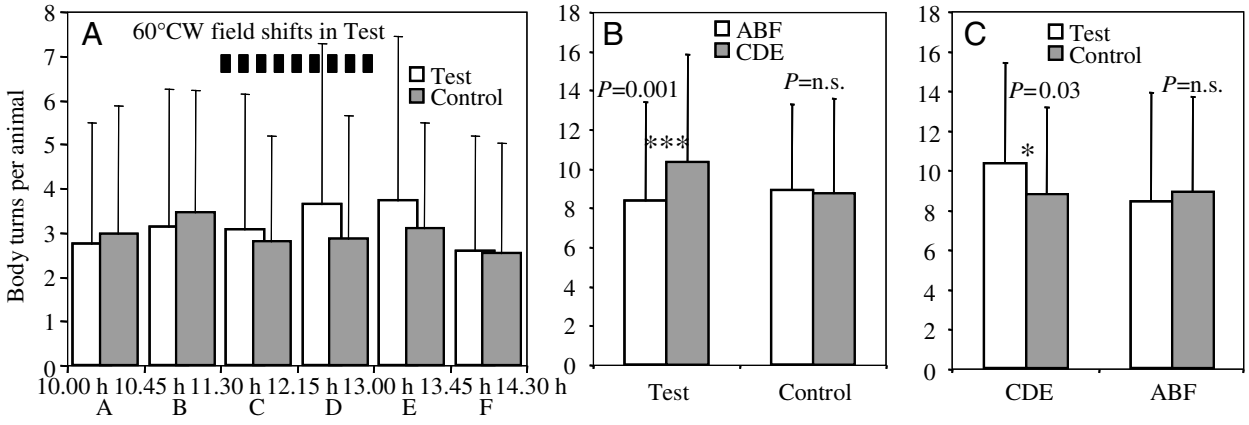

Fig. 3. Verification series. (A) The rise in activity during the CDE periods is apparent. (B) The contrast in the CDE *versus* ABF periods is significant in the Test group (Wilcoxon, N=97, P=0.001); no difference found in the Control group (Wilcoxon, N=99, P=n.s.). (C) Between the Test and Control groups, the comparison of the critical intervals CDE shows a significant difference in activity (Mann–Whitney, N=97/99, P=0.03); ABFs comparison shows no difference (Mann–Whitney, N=97/99, P=n.s.).

test and Wilcoxon test). The Wilcoxon dependent test evaluated CDE *versus* ABF individually, taking animals one by one, whereas the Mann–Whitney independent test compared the activity of test *versus* control groups without individual differentiation.

Results

In the preliminary experiment, we obtained a significant difference between the critical intervals CDE and the pre- and post-treatment intervals ABF for the test series (Wilcoxon, N=28, P=0.02) whereas the same comparison for the control showed no significant evidence of higher activity (Wilcoxon, N=28, P=0.18; Fig. 2B). Comparison of the intervals between groups by means of the independent statistics showed higher activity in the critical intervals CDE in the test which was very close to the 5% level of significance (Mann–Whitney, N=28/28, P=0.08). For the intervals ABF though, such difference was far from significant (Mann–Whitney, N=28/28, P=0.75) (Fig. 2C).

In the following verification series we obtained data repeating and confirming the trends of the preliminary test (Fig. 3B). Contrast between CDE and ABF periods is of high significance in the test group (Wilcoxon, N=97, P=0.001) but no difference was found in the control (Wilcoxon, N=99, P=n.s.; Fig. 3B). Similarly, between test and control (Fig. 3C), the comparison of critical intervals CDE shows a significant difference (Mann–Whitney, N=97/99, P=0.03), unlike the ABF comparison that shows no difference (Mann–Whitney, N=97/99, P=n.s.). Based on the data obtained, we reason that the difference between critical intervals CDE and ABF is an indicator of positive magnetosensitive reaction.

Discussion

Our test was based on the number of animal position changes when the field was rotating. Such reaction may be a consequence of non-specific discomfort due to an unstable magnetic environment, which results in higher restlessness.

Since the activity does not involve a directional component here, it represents a very simple behavioural variable. Tests of magnetoreception based on the orientation data usually involve the directional component having power to differentiate between, e.g. inclination and polarity compasses or unimodal and bimodal orientation (for a review, see Wiltschko and Wiltschko, 1995). However, the more complex the data, the more complicated their interpretation may be. For some purposes in magnetoreception research though, the directional information is not necessary and the simple analysis: magnetoreception ‘yes’ or ‘no’ may be sufficient.

Both the preliminary and the main data sets showed a remarkable delay of the magnetosensitive behavioural reaction. After the magnetic pulses are applied, the reaction needs tens of minutes to manifest fully and, in addition, it persists after the stimulus is removed. Some relevant parallels exist in the literature (both on honeybees): Hepworth et al. (Hepworth et al., 1980) found a 40–60 min latency of mobility response after the start of intermittent magnetic field stimulation (10 min intervals, vertical field, approx. 10 times stronger than the geomagnetic one). Similarly, Martin and Lindauer (Martin and Lindauer, 1977) reported a 30–45 min interval necessary for error-free dances on honeycomb after the earth’s magnetic field was artificially compensated. The interpretation of the effect remains difficult though. Whether the inertia of the behavioural responses observed is due to insect peripheral reception mechanisms, which may need a long time to become effective or whether it is a consequence of central processing of many time-dependent and multifactor inputs including animal motivation should be a subject of more specialized studies.

In terms of the testing the statistical protocol used, comparison of two time windows CDE/ABF, where the CD interval represents a 90 min period with a rotating horizontal magnetic vector, is suggested as a main diagnostic determinant.

We consider contrasting CDE/ABF intervals within individual samples stronger and more operative than test/control contrasting, which is loaded with the high inter-individual variability.

In our experiment, we are using a non-specific and non-conditioned reaction to the disturbed geomagnetic field. Conditioning tests, on the other hand, are very useful tools in insect sensory physiology research. Reward and punishment are classical instruments to provoke and fix behavioural response to the stimulus which, if alone, may provoke no measurable reaction. Nevertheless, such approach tends to be very sensitive to even small differences between both conditions during both steps – training and testing, and a risk of biasing the impact of unknown factors is high. We believe that if measurable spontaneous magnetoreceptive reaction exists, its involvement in routine magnetoreception testing may be safer in terms of successful replications than the conditioning-based protocols.

As for the model organism, we assume that there is still some shortage of routinely applicable laboratory assays on invertebrates in the realm of magnetoreception research. To date, the major interest is focused on vertebrates because of their magnetosensitive behavioural performance. However, vertebrate nervous systems are much more complex and sensitive to experimental treatment than the invertebrate one (Johnsen and Lohmann, 2005). Recent positive results on the mollusc *Tritonia,* which is a classical model for mechanisms of learning and memory studies, show a promising route for neuro-behavioural linking in magnetoreception research (Cain et al., 2005; Cain et al., 2006; Wang et al., 2004). We see our work as a contribution from the insect science side.

Although its genome sequence has not been reported yet, *Periplaneta americana* has become an important laboratory object widely used in insect neurophysiology (Comer and Robertson, 2001). In spite of the handicap of genetically poorly defined species, we believe that the assay on the American cockroach has a potential to serve as a useful tool for laboratory analysis of insect magnetoreception.

I thank Natraj Krishnan for helpful comments on the manuscript. This research was supported by a grant from the Czech Science Foundation (GACR 206/05/0911) and from the Ministry of Education, Youth and Sports (MSM 0021622416).

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