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RESEARCH ARTICLE

Cryptochrome-dependent magnetoreception in a heteropteran insect continues even after 24 h in darkness

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ABSTRACT

Sensitivity to magnetic fields is dependent on the intensity and color of light in several animal species. The light-dependent magnetoreception working model points to cryptochrome (Cry) as a protein cooperating with its co-factor flavin, which possibly becomes magnetically susceptible upon excitation by light. The type of Cry involved and what pair of magnetosensitive radicals are responsible is still elusive. Therefore, we developed a conditioning assay for the firebug *Pyrrhocoris apterus*, an insect species that possesses only the mammalian cryptochrome (Cry II). Here, using the engineered Cry II null mutant, we show that: (i) vertebrate-like Cry II is an essential component of the magnetoreception response, and (ii) magnetic conditioning continues even after 25 h in darkness. The light-dependent and dark-persisting magnetoreception based on Cry II may inspire new perspectives in magnetoreception and cryptochrome research.

KEY WORDS: Magnetoreception, Insects, Cryptochrome, Light, Behaviour, Darkness, Radical-pair

INTRODUCTION

It has been shown that animals use the geomagnetic field for orientation tasks, such as finding innate migratory directions or spontaneous directional responses in maze experiments. Magnetic cues have also been used successfully in conditioning experiments (Muheim et al., 2016; Slaby et al., 2018). Taken together, these studies show that animals can detect weak magnetic fields, but the basis of this magnetic sense is perhaps the greatest remaining mystery of sensory biology.

In more than 40 years of research, several promising tools have emerged to probe the mechanism and receptors underlying the magnetic sense. Magnetic responses in many, but not all, animals require light and are often affected by the wavelength and intensity of the ambient light (Bazalova et al., 2016; Niessner et al., 2018; Wan et al., 2021). An interplay between light and the magnetic field's effects is expected in the magnetoreception radical-pair mechanism of Schulten and Weller (1978), where light provides the energy to initiate an electron transfer and, hence, to create a magnetically sensitive radical-pair state. In the blue–green light photoreceptor cryptochrome (Cry), light absorption initiates an electron transfer cascade from nearby tryptophan residues (Trp) to the active co-factor flavin adenosine dinucleotide (FAD). FAD photoreduction triggers conformational changes, exposing the occluded C-terminal end of Cry and activating further downstream signaling responses. The link between the formation of magnetically sensitive FAD–Trp radical-pair states and molecular signaling in Cry is unique and makes it a promising magnetoreceptor candidate (Ritz et al., 2000).

Observing the magnetic responses in insect models paved the way for genetic studies demonstrating that functional Cry is indeed required for magnetic responses. Genetic removal or *Cry* expression gene silencing caused loss of magnetosensitive behavior in the fruit fly *Drosophila melanogaster* (Bae et al., 2016; Fedele et al., 2014; Gegear et al., 2008; Yoshii et al., 2009; Marley et al., 2014; Wu et al., 2016), two cockroach species (Bazalova et al., 2016) and the monarch butterfly (Wan et al., 2021). However, it is not clear whether Cry is actually a biological magnetoreceptor or instead a link in the sensory machinery downstream of an unknown receptor. Moreover, some of these genetic studies shed doubt on the role of FAD–Trp radical pairs in mediating magnetic sensitivity, suggesting instead that triggering conformational changes in Cry through any mechanism, including light-independent mechanisms, is sufficient for magnetosensitive behavior (Wiltschko et al., 2016; Hammad et al., 2020).

Furthermore, Cry proteins in animals represent several distinct types, including *Drosophila* type (hereafter Cry I) and mammalian type (hereafter Cry II). The latter, despite its name, is also found in the majority of insect species (Yuan et al., 2007), and even related species differ in having Cry I or Cry II or both (Bazalova et al., 2016; Bazalova and Dolezel, 2017). A second area of current debate is the role of Cry II in mediating magnetic field effects.

It is generally agreed that mammalian-like Cry II is light insensitive and also unable to bind FAD co-factor (Kutta et al., 2017), but the debate on FAD binding is still ongoing, especially for the subtype Cry 2 of Cry II (Hirano et al., 2017; reviewed in Vanderstraeten et al., 2020). Also, Cry II involvement in insect magnetoreception is rather contradictory and might differ between species (see Discussion). Magnetic susceptibility was rescued in *Drosophila cry I* null mutants if human *cry II* was expressed in transgenic flies (Foley et al., 2011; Fedele et al., 2014). Behavioral-genetics experiments showed that two species of cockroaches lose their magnetic sense after silencing *cry II* expression (Bazalova et al., 2016). In contrast, recently, Wan et al. (2021) showed that monarch butterflies' light-sensitive magnetoreception was fully functional even in *cry II* knockouts depending on Cry I (*Drosophila* or insect type) only.

In previous work we tested insect models expressing either only Cry II (*Periplaneta americana*) or a combination of Cry I and Cry II (*Blattella germanica*) (Bazalova et al., 2016). Here, we sought to further explore insect magnetoreception with the insect relying on

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Cry II type only in a model where stable genetic mutants had been created.

To fulfill both requirements, we established a magnetoreception assay in a new insect model, the firebug *Pyrrhocoris apterus*, a Hemipteran hemimetabolous insect widespread in Europe and western Asia.

Utilizing aversive conditioning, we developed a behavioral reaction to magnetic vector rotations. The assay follows up the magnetically induced freezing (MIF) tests used previously on *P. americana* cockroach species (Slaby et al., 2018). We found that while *P. apterus* wild-types showed MIF, the *cry II*^{04} null mutant strain was magnetically blind and this Cry-based magnetoreceptor was light dependent. Considering the recent paradigm of light dependency, we should have also observed magnetic blindness under permanent darkness. The results however, gave evidence of animals' magnetic susceptibility even after 1 day in darkness. These rather surprising results together provide support for mammalian Cry II as a molecule involved in animal magnetic susceptibility and add a new piece to the puzzle of light-dependent magnetoreception.

MATERIALS AND METHODS

Animals

We analyzed magnetoreception in *Pyrrhocoris apterus* (Linnaeus 1758), a heteropteran insect with only the mammalian type of cryptochrome Cry II (Bajgar et al., 2013). In addition to wild-type (WT) bugs, we also used *cry II* mutants engineered through CRISPR-Cas9 gene editing. In brief, the embryos were injected with *Cas9* mRNA and gRNA targeting the first exon of the *cryptochrome* gene. The mutant founder animal was identified in the F1 generation by PCR and the mutation verified by sequencing. The possible off-target mutations were outcrossed by eight generations of backcrosses to WT strains, after which the mutant line was established (for details, see Kotwica-Rolinska et al., 2019). As was shown recently, this outcrossing scheme seems to be sufficient to minimize off-targeting in *P. apterus* (Kotwica-Rolinska et al., 2020). *Cry II*^{04} contains a frameshift mutation (Kotwica-Rolinska et al., 2019; Fig. S1) which results in a premature stop codon early in the protein, therefore completely removing the CRY protein in *P. apterus cry II*^{04} (Fig. S1).

WT and mutant lines (both in the genetic background of the Oldrichovec strain) were bred in laboratory conditions. We also used *Cry II*^{04} backcrossed into the Roana strain's genetic background (Pivarciova et al., 2016). In some experiments, controls were supplemented with a WT population from Brno (Czechia).

Behavioral assay

The test was inspired by the method used in a previous *P. americana* study (Slaby et al., 2018). Briefly, MIF is a learned behavioral response to the rotation of the magnetic field (MF). MIF is induced by aversive conditioning where training consists of several cycles (see below) pairing together a MF rotation with an unpleasant stream of hot air. A trained animal displays lower motion or stops moving completely when exposed to the MF rotation alone.

Adult males and females were tested separately (Fig. 1). Each of the 16 animals used for one test was individually placed into a glass Petri dish base (diameter 8 cm). The dish base was covered with a plastic Petri dish lid, modified by cutting the surface of the lid off and replacing it with a glued on patch of common anti-mosquito plastic net, so that the bug inside was able to experience the flow of hot air from above. As firebugs are sensitive to a lack of water, they had to have constant access to it. The glass Petri dish base with the bug was inserted into another slightly larger plastic Petri dish filled with water and connected to the upper space containing a housed animal using a piece of wick string. The whole set of three dishes with the housed bug was transferred into the shielded chamber where training and testing took place.

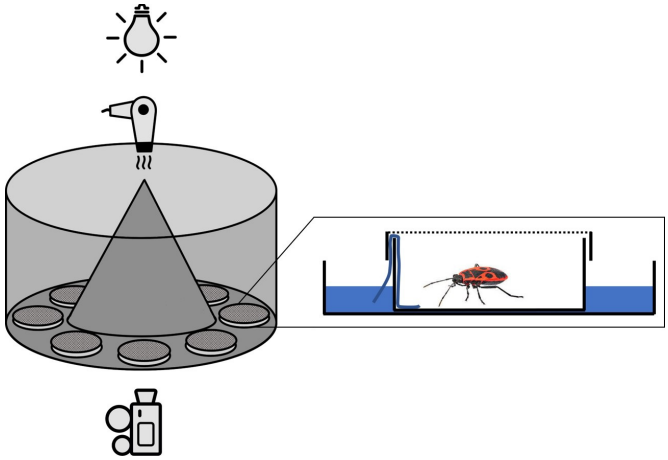

Fig. 1. Schematic drawing of the behavioral training/testing apparatus. Left: Petri dish sets containing the firebugs were placed around a plastic funnel on a glass table with a camera below. The funnel dispersed the hot air stimulus from the fan positioned above. The plastic arena encircling the ring of dishes was covered by translucent white Plexiglas which dispersed the light coming from the 505 nm blue–green LED light (for details, see Fig. S6). Right: individual firebugs were placed in a set of Petri dishes. The animals had access to water via a string wick soaked in water. The covering lid was made from a plastic net, enabling the hot air to reach the animal.

Training

Altogether, 16 dishes (Fig 1; Fig. S6) were placed on a glass pane on a non-magnetic table 100 cm above a DMK 31AU03 CCD camera (Imaging Source GmbH). Dishes were fitted into a prepared plastic template base with 16 circular holes so that the dish locations were stable and visual contacts between animals were prevented. A large plastic cone with the top upwards (diameter 30 cm) was put in the middle of the circle of 16 dishes to disperse the hot air from the fan positioned above. The space was enclosed by a white plastic arena (diameter 60 cm, height 45 cm). The arena was covered by a translucent white Plexiglas lid dispersing the light coming from the 505 nm blue–green LED light source 80 cm above the test area (see Fig. S6). In the middle of the lid, a hose leading the hot air from outside the chamber ended just above the top of the cone. A high-temperature resistant plastic hose (4 m length) led from the connector in the lid into the brass communication pipe in the shielded chamber wall where a hairdryer (1700 W) was connected to its end. The temperature of air reaching the bugs in the Petri dishes was about 60°C (Thermo-Hygrometer D3121, Comet). The arena was loaded with dishes either 1 day before the first training session in 2 day experiments or on the day of the first training session in 1 day experiments (Fig. 2). Loading for 2 day experiments took place between 15:00 h and 18:00 h, whereas loading for 1 day experiments occurred between 08:00 h and 11:00 h. These two time windows were originally settled as equally appropriate. We knew from our previous experiments (Slaby et al., 2018) that there was no difference in the conditioned MIF reaction between cockroaches waiting for the first training session, whether several hours or the whole day. Analogically, recent results under light showed that both 1 and 2 day groups performed MIF (see Results). Experiments in darkness, however, originally intended as control tests and hence comparably organized as 1 or 2 day, gave different results (see below).

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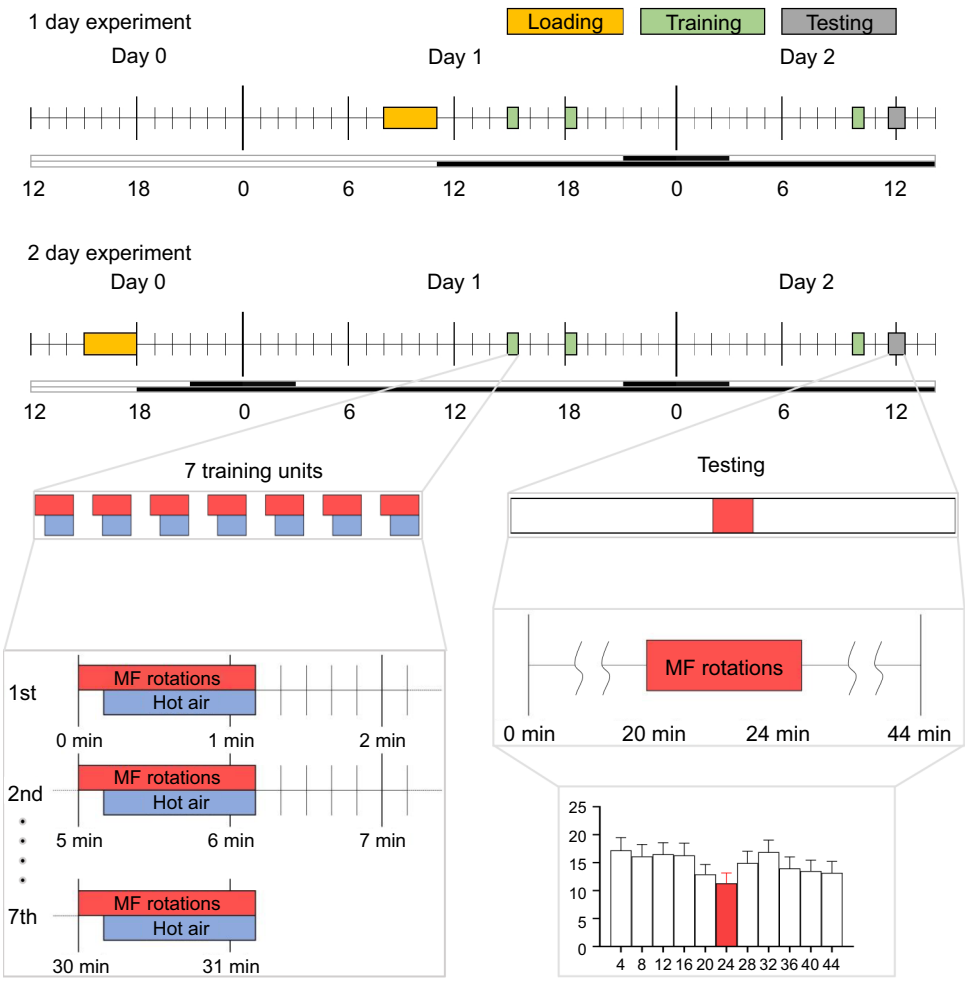

Fig. 2. Flow diagram of the experiment. Two types of tests took place: 1 day experiment and 2 day experiment, with differing time spent from loading (yellow bars) to the first training (green bars). All animals were trained and tested equally. Three training (green) blocks (21 training units in total) paired magnetic field (MF) rotations (red bars) and stream of hot air (blue bars). MF rotation onset preceded hot air onset by 10 s. The testing (gray bars) lasted 44 min and consisted of 2×20 min of steady MF prior to and after the MF rotation (24 rotations between the 20th and 24th minute). While a 18 h:6 h 505 nm light:dark regime was held in experiments under the light, no light (except IR) was present in darkness experiments (white bars indicate light, black bars indicate darkness).

The training consisted of three successive training blocks lasting 2 days: at 15:00 h, 18:00 h and 10:00 h. Each training block was composed of seven training units (Fig. 2). One training unit consisted of seven horizontal MF vector rotations (the period of rotation was 10 s) and a 60 s stream of hot air. An automatic computer-controlled system synchronized the MF rotations and hot air so that the first rotation started 10 s before the onset of the fan. Therefore, the inter-stimulation interval between the start of the conditioned stimulus (MF rotation) and the start of the unconditioned stimulus (hot air) was 10 s.

Testing

On day 2, testing started at 11:40 h and ended at 12:24 h. The rotations of the horizontal MF vector (period 10 s) lasted for 4 min in the middle of the 44 min interval (Fig. 2). Silhouettes of the firebugs were captured by a camera located underneath every 2 s until 12:24 h using IC Capture control software, AS 2.2 (Imaging Source GmbH).

Image analysis

Sets of 1321 frames from the 44 min test were automatically analyzed by Matlab-based custom-made image analysis software (available on-line at https://is.muni.cz/auth/www/vacha/roachlab_sw/). To detect motoric activity, the number of body shifts >0.5 cm and/or turns of body axis >50 deg was calculated, resulting in a record of moves for each animal.

We did not check the age of the animals entering the test. As generations alternated, some groups showed more dead animals at the end of the test and generally lower activity than other groups. Hence, we discarded data from old and/or dying animals with extremely low activity according to the following rules. Individual samples were discarded if an animal moved altogether 3 times or fewer during the test (16% of individuals were discarded). The whole sample of 16 animals was discarded if there were more than 6 animals with 10 movements or fewer (17% of groups were discarded). This selection was done automatically regardless of the sample type.

Experimenter blindness concerns

Diverse sample groups (WT, mutants, control males, females, etc.) were alternated randomly (see the complete list of primary data: https://is.muni.cz/www/vacha/supplementary_materials_netusil_2021/Linden_Bug_Magnet_Primary_data.xlsx). Individuals from different groups were tested together and the person evaluating the data was not aware of how samples he/she was processing were mixed together. The experimenter who prepared the testing and analyzed the data was not aware of what wiring for the double-wrapped coils (counter-current or normal) was selected. In some cases (1 or 2 day experiments), the experimenter was not blind as to what sample he/she was processing but the data were analyzed exclusively and completely automatically by the image analyzing software with all parameters permanently set.

Statistics

For each animal, the number of movements per minute during the 4 min period of rotation was compared with the number of

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movements per minute during the 40 min period before and after the rotation. A paired, two-tailed *t*-test was applied to compare the animals' activity during 'Steady' and 'Rotation' states (see Figs 3–5). To circumvent the problem of having non-parametrically distributed individual differences, we used a resampling permutation technique.

MF conditions

A constant static MF was set permanently inside the electromagnetically shielded chamber, with total vector 50 µT (space deviation ±0.5 µT) and inclination 0 deg (measured by FGM3D/100 magnetic field sensor; SenSys). If a rotating MF was set, the horizontal vector 50 µT rotated clockwise for a 10 s period (deviation ±1 µT, inclination 0 deg). MF vectors were generated by 3D Merritt coils (2.5×2.5×2.5 m) located inside the chamber (Fig. S6) and fed by custom-made, computer-controlled power supplies operating on a D/A interface (National Instruments). The coil feeding unit was located outside the chamber and connected to Merritt coils through filters (6EMC1, Corcom).

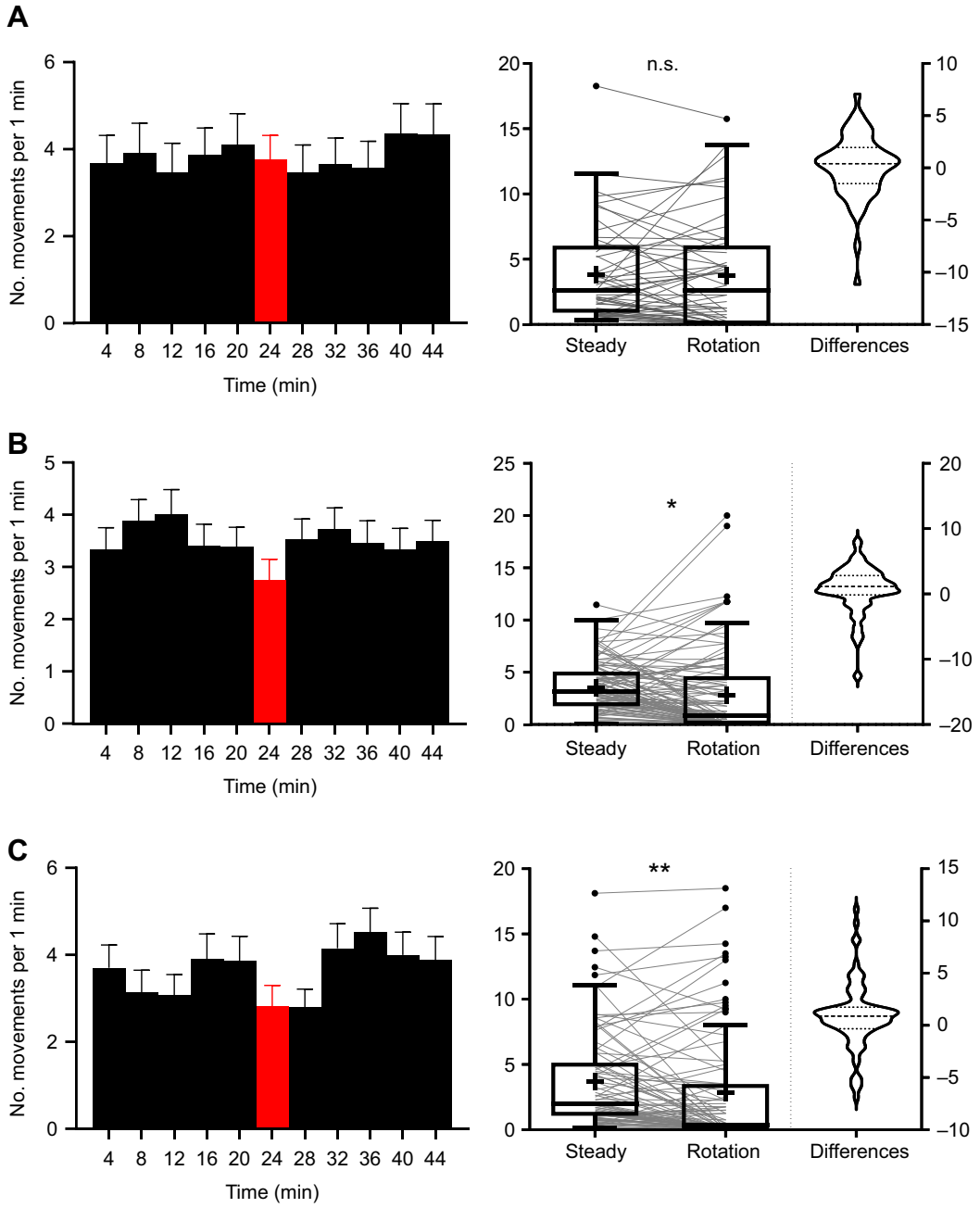

Fig. 3. Wild-type firebugs of both sexes tested in blue–green light. (A) Females did not show conditioned magnetically induced freezing (MIF) (n=54, P=0.4340). Males showed MIF in both (B) 1 day (n=104, P=0.0142) and (C) 2 day (n=88, P=0.0044) experiments. Left: mean±s.e.m. number of movements per 1 min. The black 4 min bars represent periods of steady MF; the red 4 min bar represents the period of MF rotation. Right: box plots depict the mean activity per minute for the two conditions ('steady' and 'rotation'), with the individual pairs connected by a gray line from the same data. The box plots show the median, 25th and 75th percentiles, Tukey whiskers and outliers (means are given as crosses). Distributions of individual differences are shown on the extreme right. As normality was not secured, a paired, two-tailed *t*-test in combination with a permutation test was applied. *P<0.05, **P<0.01.

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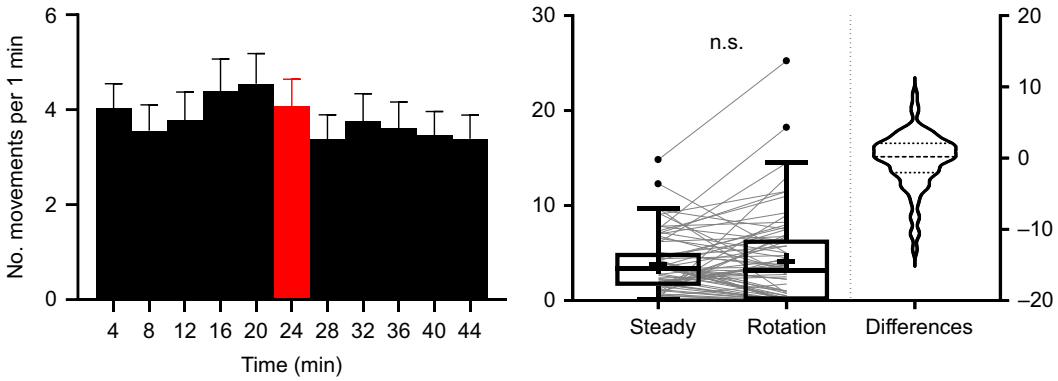

Fig. 4. Males from the *Cry II*^{04} mutant line tested in blue–green light. MIF was not apparent (n=72, P=0.7291). Diagrams and descriptions above.

Radiofrequency background in the chamber

For background radiofrequency electric and MF spectra, see Fig S4. A spectrum analyzer (FSC3, 9 kHz–3 GHz, Rohde and Schwartz) and active Schwarzbeck HFS 1546 antenna were used to measure the magnetic component of radiofrequency noise. An active Schwarzbeck EFS 9218 antenna was used to measure the electric component of radiofrequency noise.

Illumination

Darkness or blue–green light (505 nm) was used and checked prior to the experiment using a radiometer (International Light IL700, SHD 033 probe). Blue–green 505 nm LED (SELV CK7P) illumination at 5.3×10^{-6} W cm^{-2} varied from maximum to minimum inside the arena by 0.6×10^{-6} W cm^{-2}; LED spectrum distribution was measured with a USB 2000 spectrometer (Ocean Optics). The light:dark regime both in the stock room and in the shielded chamber was 18 h:6 h in the illuminated experiments. For the experiments in darkness, the light was permanently switched off from loading until the end of testing. An infra-red LED lamp (852 nm) illuminated the arena from above to make the firebugs visible for the camera. The white ceiling light illuminated the animals when loading of the dishes occurred in the shielded chamber. This intensity was 2.5×10^{-4} W cm^{-2} (SKE 510, Skye Instruments). All light spectra are given in Fig. S5.

RESULTS

First, we addressed whether the firebugs could perform MIF as a result of aversive conditioning pairing of MF rotation and hot air. Animals of both sexes were trained and tested under 505 nm blue–green light. Samples showed insignificant MIF in females (n=54, P=0.4340) (Fig. 3A) and significant MIF in males (male 1 day test: n=104, P=0.0142; and male 2 day test: n=88, P=0.0044) (Fig. 3B,C, respectively). Hence, sex influenced the final phenotype.

To see whether MIF is a true conditioned response to MF rotation, we performed several control experiments where training, testing or both were skipped. We monitored the following combinations: spontaneous male activity with no training and no MF rotation during the test (n=78, P=0.7360) (Fig. S2A), untrained male activity under application of the test only (n=64, P=0.7205) (Fig. S2B), trained male activity, and no MF rotation during the test (n=60, P=0.7535) (Fig. S2C).

We also performed analogical control experiments on females, where two combinations were monitored: spontaneous female activity with no training and no MF rotation during the test (n=66, P=0.3567) (Fig. S3A), and untrained female activity under application of the test only (n=61, P=0.5164) (Fig. S3B).

Neither of these control experiments gave significant MIF, altogether showing that there was no spontaneous increase or decrease in activity in either of the untrained sexes at the time when the critical 4 min period was monitored. We also learned that fully trained males do not change this steady behavioral pattern at the time when the critical 4 min period is monitored. Further, we can conclude that there was also no increase or decrease in activity after 4 min of MF rotation in untrained animals of both sexes.

In our following tests, we focused on males only. To investigate the impact of different periods spent in dishes waiting for the first training unit, we separated samples loaded into the arena on the morning before the training (male 1 day test: n=104, P=0.0142) (Fig. 3B) from the samples loaded into the arena on the afternoon of the day before the training (male 2 day test: n=88, P=0.0044) (Fig. 3C). The MIF in each of the variants was significant. Therefore, we concluded that the time spent in dishes under blue–green light from the moment of loading to the end of testing – approximately 28 or 45 h – had no impact on MIF performance.

In the next step, we investigated whether MIF is dependent on Cry II. We employed Cry mutants engineered with CRISPR-Cas9 gene editing. The *Cry II*^{04} mutant line did not show significant MIF (n=72, P=0.7291) (Fig. 4). Consequently, Cry II was shown to be indispensable for magnetoreception of *P. apterus*.

As the last step, we included a control test for the light-dependent magnetoreception phenotype in darkness. We expected a loss of magnetoreception as we had experienced in our previous laboratory experiments performed in darkness (Bazalova et al., 2016; Bartos et al., 2019). However, in contrast to pilot experiments with both sexes, we obtained rather ambiguous results showing possible magnetic sensitivity, even in darkness. Having tested samples separated according to the time spent in darkness prior to the training and testing, both in darkness, we obtained two different results: the firebug males that had spent almost 2 days (from 42 to 45 h) in darkness could not detect MF rotations (n=81, P=0.3007) (Fig. 5A), but males that had spent about a day in darkness (from 25 to 28 h) could still detect it (n=73, P=0.0012) (Fig. 5B). Considering they had been illuminated from the chamber ceiling lights tens of hours ago, this ability was surprising. To exclude the possibility that animals were trained to some unperceivable non-magnetic stimulus instead of the horizontal MF rotation, we performed a 1 day experiment in darkness while counter-current electrical feeding the Merritt coils using double-wrapped wiring. As MIF was not detected (n=69, P=0.4221) (Fig. 5C), we conclude that MF rotation was the only sensory stimulus causing MIF.

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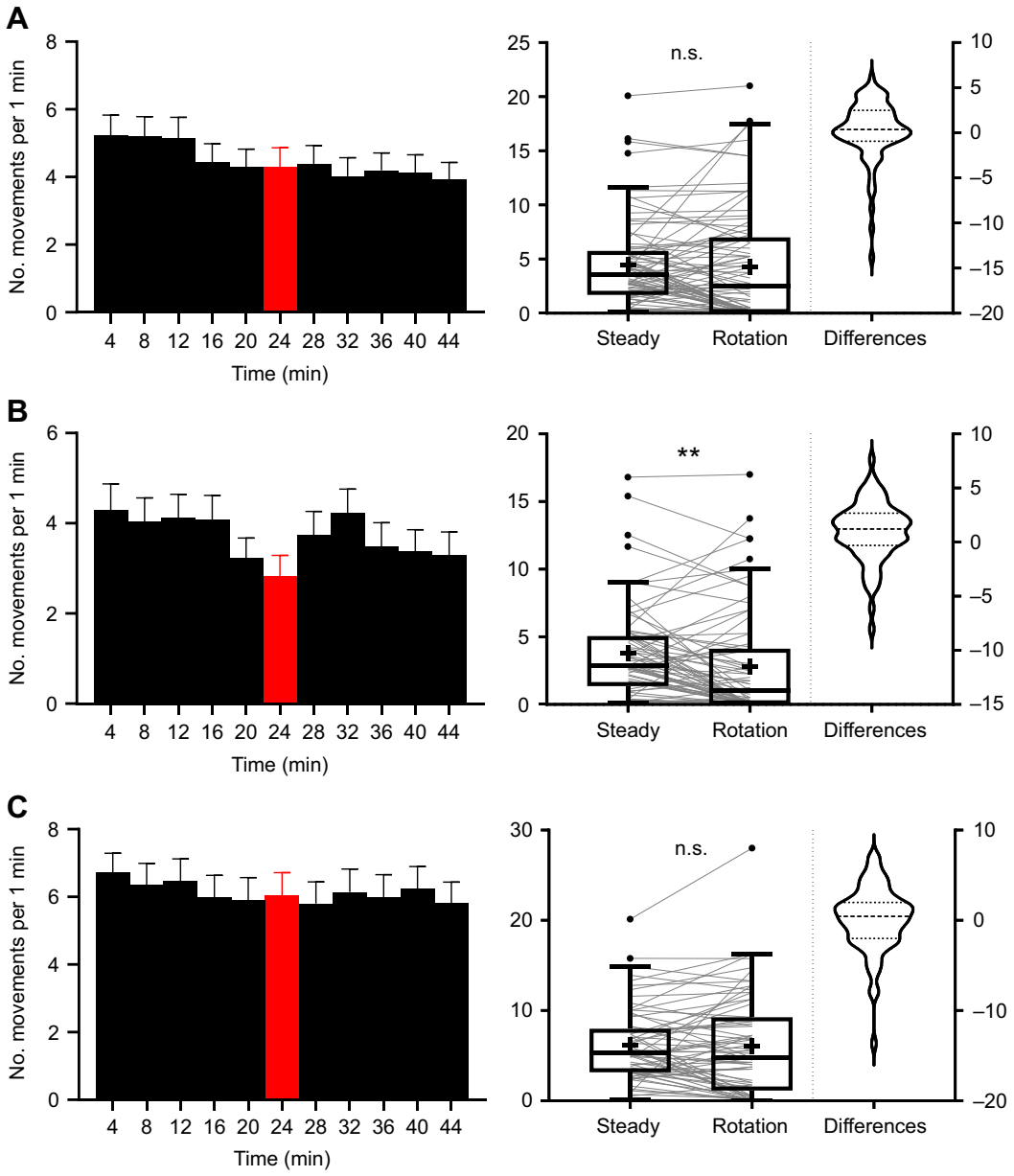

Fig. 5. Wild-type males tested in darkness. (A) No MIF is apparent after 2 days in darkness (n=81, P=0.3007); but (B) after 1 day in darkness MIF is significant (n=73, P=0.0012). (C) If the same 1 day experiment is performed with counter-current coil wiring, no MIF takes place (n=69, P=0.4221). Diagrams and descriptions above.

DISCUSSION

Involvement of mammalian Cry II in magnetoreception

The study's findings contribute to the ongoing discussion on the possible roles of mammalian Cry II in insect magnetoreception. Here, Cry II was shown to be required for magnetoreception of *P. apterus*, a species expressing solely this type of Cry. This finding is in line with our previous data from cockroaches losing spontaneous magnetoreception behavior after silencing *Cry II* by RNAi (Bazalova et al., 2016). *Cry II* was also shown to rescue magnetoreception in *Drosophila* genetic constructs where the missing gene for *dCry* (encoding *Drosophila* Cry protein) was replaced by *Cry II* (Foley et al., 2011; Fedele et al., 2014). However, some insects apparently do not employ Cry II for their magnetoreception, as recently shown for the monarch butterfly (Wan et al., 2021). Monarch *Cry II* knockouts responded to magnetic stimuli while *Cry I* knockouts did not.

The situation is somewhat similar to the role of Crys in the insect circadian clock. Whereas some species, such as the monarch butterfly or the bean bug (Ikeno et al., 2011; Zhang et al., 2017), possess Cry II, which is essential for the clock 'ticking', *Drosophila* contains only the Cry I type, which is primarily involved in the light-mediated resetting of the clock (Emery et al., 2000). Interestingly, flies genetically depleted of Cry I display aberrations in the circadian clock at high and low temperatures (Dolezelova et al., 2007).

Furthermore, Cry is essential for proper circadian clock function in the peripheral tissue of *Drosophila*, where Cry I depletion abolishes cyclical expression of circadian reporters (Stanewsky et al., 1998; Collins et al., 2006). Apparently, even the role of Cry I

in *Drosophila* differs in a tissue- and temperature-dependent manner.

Stability of radical-pair cycle intermediates and the question of darkness

Light has been considered one of the key factors distinguishing between radical-pair hypotheses of reception and an alternative hypothesis of light-independent reception built on ferrum oxide (magnetite) particles (Eder et al., 2012). Here, we show that in *P. apterus*, magnetoreception is both Cry dependent and functions after tens of hours in darkness. Our results do not contribute to the discussion on whether Cry is the primary receptor or not. As magnetic susceptibility is lost in darkness some time between 28 and 45 h after the last illumination, we conclude that it is still light dependent and, hence, not compatible with the magnetite magnetoreception hypothesis. How compatible, however, are our results with the radical-pair magnetoreception model?

If a magnetically sensitive radical-pair step occurs in the reactions following Cry activation by light, then it is possible to have magnetic sensitivity in darkness as long as a population of activated Cry molecules is present. Several cases of animal and plant magnetoreception have indeed been shown to be dependent on initial illumination but with the magneto-sensitive step itself taking place in darkness. When MFs were only present during dark periods of 0.7 s following 0.3 s periods of illumination, European robins oriented normally to MFs (Wiltschko et al., 2016). Cry-dependent magnetic sensitivity in darkness was reported for the *Arabidopsis* plant with a 10 s interval between the end of the light pulse and the onset of the MF (Pooam et al., 2018) and when exposure to MF was provided in 10 min dark intervals only (Hammad et al., 2020).

In the Cry photocycle, initial light absorption leads to the formation of a relatively stable FADH° redox state, considered the active state for Cry signaling. In birds, immunohistological studies indicate that this active state persists for tens of minutes but is depleted after 1 h (Niessner et al., 2014). This time scale is matched by behavioral observations of birds orienting well under green light within tens of minutes of initial illumination, but not after 1 h of darkness (Wiltschko et al., 2014).

There is a conceptual similarity between the results presented here and in the above-mentioned studies in that magnetoreception can occur in the dark after initial illumination, up to a time limit in darkness beyond which magnetoreception is abolished. However, the time limit found in our experiments with magnetoreception persisting after tens of hours of darkness has no precedent in the literature. It is possible that extraordinarily long-lived redox Cry intermediates persist and unconventional radical-pairs might be employed during the dark phase of redox cycle in firebugs. Or, alternatively, firebug Cry may be activated in the absence of short wavelength light.

The question arises whether the persistence of light-dependent magnetic sensitivity into the dark phase is a general phenomenon in insects. At the very least, our results show that previous interpretations concerning the reception mechanism of some seemingly light-independent experiments might need to be reconsidered. The analogical problem should also be discussed: why other experimental results showing Cry dependency and light dependency do not persist into the dark phase and animals lose their magnetoreception if the light is turned off within a time much shorter than tens of hours (Bazalova et al., 2016; Bae et al., 2016; Wan et al., 2021). One reason for the difference may lie in the different motivations of the animals tested in spontaneous and conditioned paradigms. In previous tests, animals might not show the same spontaneous magnetosensitive behavior when the light was off as they did when the light was on, even though the underlying biophysical mechanism for magnetic sensing may still operate. Darkness may switch from a 'daylight behavioral program' to a different program. In contrast to cited works, our recent testing is based on conditioning, and animals are trained and tested under identical light conditions and so are unable to differentiate among training and testing; therefore, their motivation is not biased.

Imperfect darkness and the impact of IR light

Our experiments were not performed in absolute darkness as animal movements were captured by camera and the scene had to be illuminated by an IR lamp. In behavioral experiments on insect magnetoreception, IR light (>800 nm) has been assumed to be invisible for insects (Briscoe and Chittka, 2001) and to not interfere with the magnetoreception mechanism. As no redox form of flavin absorbs IR light, long wavelengths have not been considered relevant and IR light was used as necessary lightening for cameras in tests designated as 'darkness' (Bazalova et al., 2016; Wan et al., 2021). If, however, the energy of IR light induced – via an unknown mechanism – (re)creation of radical-pair redox cycle excited states, then it would have had consequences for the setup of the behavioral experiments under the condition of 'darkness'. In our work, we did not test the possible impact of IR on magnetosensitive behavior specifically. Nevertheless, MIF under IR light is time limited in contrast to MIF under blue–green light. Therefore, we conclude that even if IR prolonged the period of magnetic susceptibility when no visible light was present, it is not sufficient to keep the system working permanently and, thus, is dependent on wavelengths shorter than IR.

Sex-dependent behavior

We found a difference in MIF between males and females. The sex apparently influences the MF perception process or animals' learning or motivation to react. The phenomenon of sexual dimorphism is not new within magnetoreception tests in insects. In the fruit fly, Phillips and Sayeed (1993) found a learned magnetic compass response only in adult males. Similarly, Oh et al. (2020) report sex-dependent magnetic imprinting behavior in fruit flies and Wan et al. (2020) report different behavioral and physiological reactions to MF between sexes in the brown planthopper, *Nilaparvata lugens*.

Our work introduces a new insect species employing solely mammalian Cry II as part of its magnetoreception system. The assay may contribute to the ongoing discussion on the role of Cry II in magnetic sensitivity in general. Additionally, the work demonstrates that light-dependent magnetoreception based on Cry may persist for tens of hours in darkness, which has consequences for the design of experiments. The findings may open the door to unexpected solutions in the search for magnetically sensitive photochemical reactions in living organisms.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.N., T.R., M.V.; Methodology: R.N., L.C., D.C., D.D.; Validation: R.N., K.T., M.V.; Formal analysis: R.N., L.C., D.C., D.D.; Investigation: R.N., K.T.,

L.C., D.C., M.V.; Data curation: L.C., D.C., D.D., M.V.; Writing - original draft: R.N.; Writing - review & editing: D.D., T.R., M.V.; Supervision: D.D., T.R., M.V.; Project administration: M.V.; Funding acquisition: D.D., M.V.

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Data availability

The complete list of primary data is available online at: https://is.muni.cz/www/vacha/supplementary_materials_netusil_2021/Linden_Bug_Magnet_Primary_data.xlsx

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