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Experienced migratory bats integrate the sun's position at dusk for navigation at night

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HIGHLIGHTS

- First effective manipulation of the compass system in a migratory mammal
- Adult bats shifted orientation at night by misinterpreting a mirrored sun at dusk
- There may be no innate component to orientation in first year migrants
- A novel assay to measure bat takeoff orientation based on free directional choice

In Brief: Navigation of migratory mammals remains largely understudied due to the lack of a controlled behavioural assay for measuring orientation. Here, Lindecke and colleagues have developed an experimental behavioural assay for migratory bats to test the role of the sun's position in the compass system by measuring their takeoff orientation. Takeoff behaviour suggests that adults calibrate their compass with the sun's azimuth at sunset, but that first year migrants appear unable to take up a migratory heading, suggesting that migration direction is learned rather than innate in these bats.

SUMMARY

From bats to whales, millions of mammals migrate every year. However, their navigation capacity for accomplishing long-distance movements remains remarkably understudied and lags behind by five decades compared to other animals [1, 2]; partly because, unlike for other taxa such as birds and sea-turtles, no small scale orientation assay has so far been developed. Yet recently, bats became a model to investigate the nature of the cues mammals use for long-range navigation, and surprisingly for nocturnal animals, sunset cues, and in particular polarized light cues, appear crucial to calibrate the magnetic compass system in non-migratory bats [3–5]. This does not appear to hold for a species of migratory bat however [6], and thus the nature of the information used by migratory bats for navigation remains unclear. In this experiment, we asked whether the position of the solar disk *per se* is relevant for compass orientation in a migratory bat, *Pipistrellus pygmaeus*. Using a new experimental assay that measures takeoff orientation, we tested the orientation of bats exposed to a shifted sunset azimuth using a mirror at dusk. Bats exposed to a 180° rotated azimuth of the setting sun and released after translocation during the same night shifted their heading direction by ~180° compared to control bats. However, first-year migrants had no clear orientation either as controls or following the same treatment, indicating that unlike birds, these bats do not have an innate migratory direction when released after translocation. This suggests learning is a key component in the long-range navigational system of naïve bats in this species. Our study provides rare empirical evidence for the specific cues and mechanisms migratory mammals use for navigation.

Keywords: animal migration, bats, compass calibration, orientation, magnetoreception, solar orientation, takeoff behaviour

RESULTS AND DISCUSSION

Migratory animals show an ability to navigate over vast distances with remarkable precision returning to the same area year after year [7]. Whilst some aspects of this behaviour remain a mystery, advances in our understanding have been made in taxa such as birds and reptiles, by using small scale correlates of orientation that have allowed controlled manipulation of the cues animals are using [8, 9, 10]. One taxon, mammals, remains remarkably understudied with regard to navigation during migration however. Whilst the logistics of experiments on large terrestrial and aquatic migrants contributes to this dilemma, migratory insectivorous bats also remain understudied. A key reason for the lack of research on bats is the absence of an assay that measures a correlate of migratory orientation such as those that exist in birds and reptiles [1]. Therefore, a thorough understanding of the senses and environmental cues used by mammals for navigation over hundreds or even thousands of kilometres is lacking [11]. Bats have emerged as a widely studied model in movement ecology because they combine both high ecophysiological diversity with a variety of movement behaviours [12]. Bat eyes evolved to sense a wide range of light and a broad spectrum of wavelengths [13, 14] and, presumably, bats of the family Vespertilionidae, and possibly also others, rely heavily on vision when orienting over long distances since echolocation and path integration are ineffective and error-prone at distances larger than a few dozen meters [15–19].

Generally, the sun is considered to be the most prominent celestial cue for compass orientation, since it is key for bird navigation during homing and migration [20, 21]. Surprisingly, even nocturnal mammals, such as bats, integrate solar cues to successfully find their home roost at night, which appears counterintuitive because of bats' strictly nocturnal behaviour [4, 5, 22]. Furthermore, it has been demonstrated that adult non-migratory bats calibrate a magnetic compass to cues at sunset [3, 4]. Like some migratory birds [23], a non-migratory bat species was demonstrated to use the maximum band of polarized sunlight at sunset for this calibration, which forms a band across the sky at 90° to the setting sun [5]. In contrast, however, the only experiment so far to test the cues used for navigation by a migratory bat species, namely Nathusius' bats (*Pipistrellus nathusii*), failed to find an effect of manipulation of the polarized light pattern on heading directions [6]. Therefore the cues and mechanisms used by migratory bats during navigation remain entirely unknown [24].

Here, we tested whether the position of the setting sun on the horizon (solar disc azimuth) could be the key reference for bats to efficiently calibrate their compass system for navigation at night. We caught fifty-four Soprano pipistrelles (*Pipistrellus pygmaeus*) migrating towards their wintering grounds along the Latvian Baltic Sea shore. We discriminated between first-year migrants and adult bats, assuming that subadults are inexperienced and adults experienced migrants. After capture, bats were housed in small groups in a dark keeping room until the next evening. Our experimental approach was based on two-steps: (1) exposing bats to a shifted position of the sun at sunset created by using a polished steel-mirror and (2) subsequent translocation of experimental animals further inland with an orientation test at the release site during the same night. For the sunset treatment, we chose a location in the dunes with an open view of the Baltic Sea. On the day of treatment, bats were randomly assigned to two treatment groups and individually put in cylindrical cages with lids limiting their free view to the natural (C) or mirrored (M) sunset from 30 min before until 30 min after sunset, i.e., until the sun's disk disappeared below the horizon (-5°). For each treatment group, the opposite view of the horizon was blocked from vision for 180° by the taped sidewall of the cage. Thus, bats of the M group faced the mirror 50 cm in front of them instead of a free view of the forest to the east opposite to sunset. At the treatment site, we took utmost care that bats were not exposed to any other light source than defined by the experimental condition, i.e., we abstained from using artificial lights when collecting the animals from their cages again. We hypothesized that migrating bats use the sun, i.e. the directional information provided by the solar disk at sunset, to calibrate their orientation system. Accordingly, we expected bats of the M group to orient in the opposite direction compared to bats of the C group when released.

To mirror the sunset, we used pure metal plate mirrors (stainless steel, 60×60 cm) as recommended for deflector studies by Horváth & Pomozi (1997) [25]. Using metal plate mirrors is crucial since common glass mirrors generate unwanted optical stimuli with qualitative and quantitative changes of the natural skylight polarization and near ultra-violet light patterns which have been shown to bias animal orientation in deflector studies [25, 26]. When handling bats, we ensured that they neither saw the sky nor the true horizon before and after the experimental treatment. After exposure to the sunset-treatment, we translocated the bats 5.1 km inland for nightly release on a large meadow surrounded by trees.

For recording of the heading directions of released bats, we designed a behavioural assay to quantify the departure flight direction based on measures of takeoff using a novel apparatus, a

circular release box for bats (CRBox). The CRBox allowed us to remotely release bats and record their takeoffs in every possible compass direction (Figure 1). The CRBox consists of a circular-shaped arena with a lid including the remotely controlled mechanism for release of a bat from the centre of the arena. The apparatus is positioned in the field 1 meter above ground. By construction and due to translocation away from the treatment site, the CRBox reduces the amount of environmental information which could inform the navigation system of a bat, e.g., cues provided by the night sky, visual landmarks and winds. From the moment of release, the echolocation behaviour of the bat in the CRBox was monitored with the aid of a bat detector from 10 m distance. When echolocation calls indicated flight by characteristic regular pulses, we noted the timing of takeoff. A 360° thin layer of chalk on the annulus of the arena enabled us to record the animals' orientation based on tracks, i.e., the footprints left after crawling to the edge for takeoff. This experimental behavioural assay is based on the recent finding that takeoff orientation is a reliable proxy for departure flight orientation in adult bats [27].

We found that translocated adult bats took off for departure in a direction that depended on the experimental treatment. The heading direction depended on the sunset azimuth that bats had been exposed to, and which they apparently integrated for navigation decisions. Mean takeoff orientation of bats previously watching the natural sunset averaged 256° (west-southwest) in the C group, and 79° (east) in the M group which matched the 180° difference in the perceived azimuth of the sun during the treatment. Orientation data of both groups were significantly different from a uniform circular distribution (Kuiper test, adult C: $p < 0.01$, $V = 2.04$, $n = 11$; adult M: $p < 0.025$, $V = 1.977$, $n = 15$; Figure 2), both being significantly unimodal (Rayleigh test, adult C: $r = 0.591$, $p = 0.018$, $Z = 3.848$; adult M: $r = 0.511$, $p = 0.017$, $Z = 3.915$). The variance of individual orientations around the group-specific means did not differ significantly, and thus can be considered equal (Levene's test: $F_{1,24} = 0.061$, $p = 0.806$), which is an additional indicator of the effectiveness of the mirror treatment. Distributions were significantly different between groups (Mardia–Watson–Wheeler test, $W = 11.238$, $p = 0.004$) while both groups took off in opposite directions (177° difference of mean angles). During 19 releases, unambiguous echolocation behaviour could be observed, indicating the exact moment when focal bats took off for flight. There was no significant difference in latencies between groups (adult C: mean = 88 sec, median 72 sec $n = 7$; adult M: mean = 85 sec, median = 81 sec, $n = 12$; $t = 0.177$, d.f. = 17, $p = 0.908$).

In contrast to adult takeoff orientations, subadult bats ($n = 24$) did not show any directional preference, displaying random orientation in both control and experimental groups. Takeoff data distributions from subadult control (subadult C) and mirrored (subadult M) bats did not differ from a uniform circular distribution (Kuiper test, subadult C: $p > 0.15$, $V = 1.422$, $n = 14$; subadult M: $p > 0.15$, $V = 0.952$, $n = 10$; Fig. 1), and thus any unimodal orientation could be ruled out a priori, which is supported by Rayleigh test results (subadult C: $r = 0.313$, $p = 0.258$, $Z = 1.369$; subadult M: $r = 0.801$, $p = 0.801$, $Z = 0.232$). In accordance with the above tests, we found no significant difference in either the variance of individual headings around group-specific means (Levene's test: $F_{1,22} = 1.328$, $p = 0.262$), nor the distributions of both groups (Mardia–Watson–Wheeler test, $W = 0.813$, $p = 0.666$). Thus, first year migrants were not oriented irrespective of treatment. Again, takeoff latencies did not differ between treatment groups (subadult C: mean = 74 sec, median = 68 sec, $n = 11$; subadult M: mean = 77 sec, median = 64 sec, $n = 7$; $t = -0.139$, d.f. = 16, $p = 0.891$), nor was there a difference when we compared mean latencies among the age groups, suggesting that in principal subadults behave similarly when compared with adult test bats (adult C & M: mean = 86 sec, median = 72 sec, $n = 19$; subadult C & M: mean = 75 sec, median = 66 sec, $n = 18$; $t = 0.693$, d.f. = 36, $p = 0.493$). Interestingly, in other taxa time-compensated sun compass orientation is possible at an early stage of ontogeny, as it supports long-distance dispersal movements of diurnal fish larvae and juvenile sea turtles [28, 29]. If so, this suggests that unlike many first year migratory passerines which migrate alone, and are able to count on genetically controlled population specific migratory orientation in the wild [30], and in an orientation cage [31], these bats do not appear to have an inherited migratory direction calibrated by the sun, or at least are not able to express it in the apparatus we use. This suggests that young *P. pygmaeus* need to learn long-distance orientation *en route* of migration from conspecifics, similar to some long lived bird species [32, 33].

In conclusion, our study provides the first evidence to show unambiguously that a migratory bat species integrates the direction of the setting sun's disc at dusk to calibrate a compass system for nocturnal orientation, a capability which to our knowledge has not been shown for any other animal species. This complements recent results which did not support a calibration based on polarized light cues in migrating pipistrelle bats (*P. nathusii*), suggesting that magnetic compass calibration is dependent on the solar azimuth at sunset. However, we did not track the migratory transit flight as such and thus cannot exclude actively migrating bats use different navigation

strategies, such as route-following to complement map-and-compass navigation where possible. Also, it cannot be excluded that bats use solar azimuth-based orientation not only in the migration context but in a more general way, as well. For example, heading back to any flight corridor after natural displacements resembles the situation which we have observed in our experiment, which again is similar to the navigational challenge of locating foraging grounds or home roosts outside the migration season. To date, it remains unknown how subadult *P. pygmaeus* navigate on their first migration. We speculate that subadults may learn by social factors since we regularly observe groups of 2 to 3 bats passing by the migratory corridor at our study site [12]. Our results highlight for the first time in a migratory mammal that first year migrants appear to lack an inherited migratory direction and thus may rely on travelling with experienced conspecifics when making their first migratory journey – an intriguing difference to other migrants, such as juvenile passerine birds and sea turtles [9, 30, 34].

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Author contributions

O.L. developed the methodology and conceptualised the study. O.L. and A.E. carried out the experiment; C.C.V. and G.P. supported fieldwork. O.L. conducted the analyses, and drafted the original manuscript. C.C.V. and R.A.H. reviewed and edited the manuscript; all authors commented on the manuscript.

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Figure 1. The bat takeoff orientation assay. Scheme showing a bat departing from a circular release box. After the acclimatisation box is lifted, the animal may leave from the apparatus at the time and in the direction of choice. A thin layer of chalk tracks the movements of bats while orienting and crawling to the opening, enabling an accurate measure of takeoff orientation. The input of environmental cues is reduced until the bat takes off for free flight.

Figure 2. Nightly takeoff orientation of adult and 1st-year bat migrants (*Pipistrellus pygmaeus*) at release depending on preceding sunset direction. Arrow and dashed lines (95% confidence intervals) highlight significant unimodal directionality in orientations of adult groups (A, B; $n = 26$) according to the Rayleigh test (5%, p -values are shown in the circular graph). The length of the black arrows corresponds to the vector length, r , in each graph. Orientation data of both subadult groups (C, D; $n = 24$) were indistinguishable from random distributions (Kuiper test results: $p > 0.15$). *MWW.p* and *Levene's.p* are p -values of the Mardia-Watson-Wheeler and the Levene's tests respectively, performed between neighbouring treatment groups. While adults but not subadults differed in distributions (*MWW*), the scatter of data around the means was equal (Levene tests). Significant differences are highlighted in bold. Total sample size $n = 50$.

Figure 3. Experimental cage used to expose bats to the natural or mirrored sunset. The cage design offers a 180° free panoramic view.

Data S1. Raw data of bat releases and heading data used in the statistical analysis.

Figure 1.

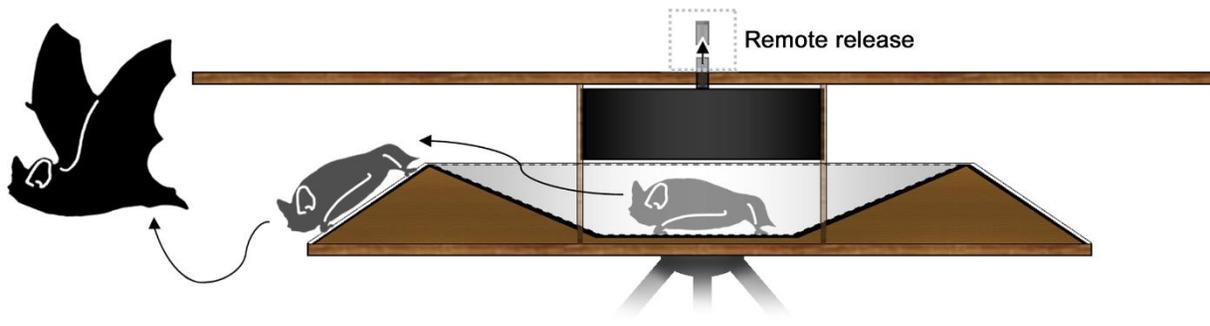


Figure 2.

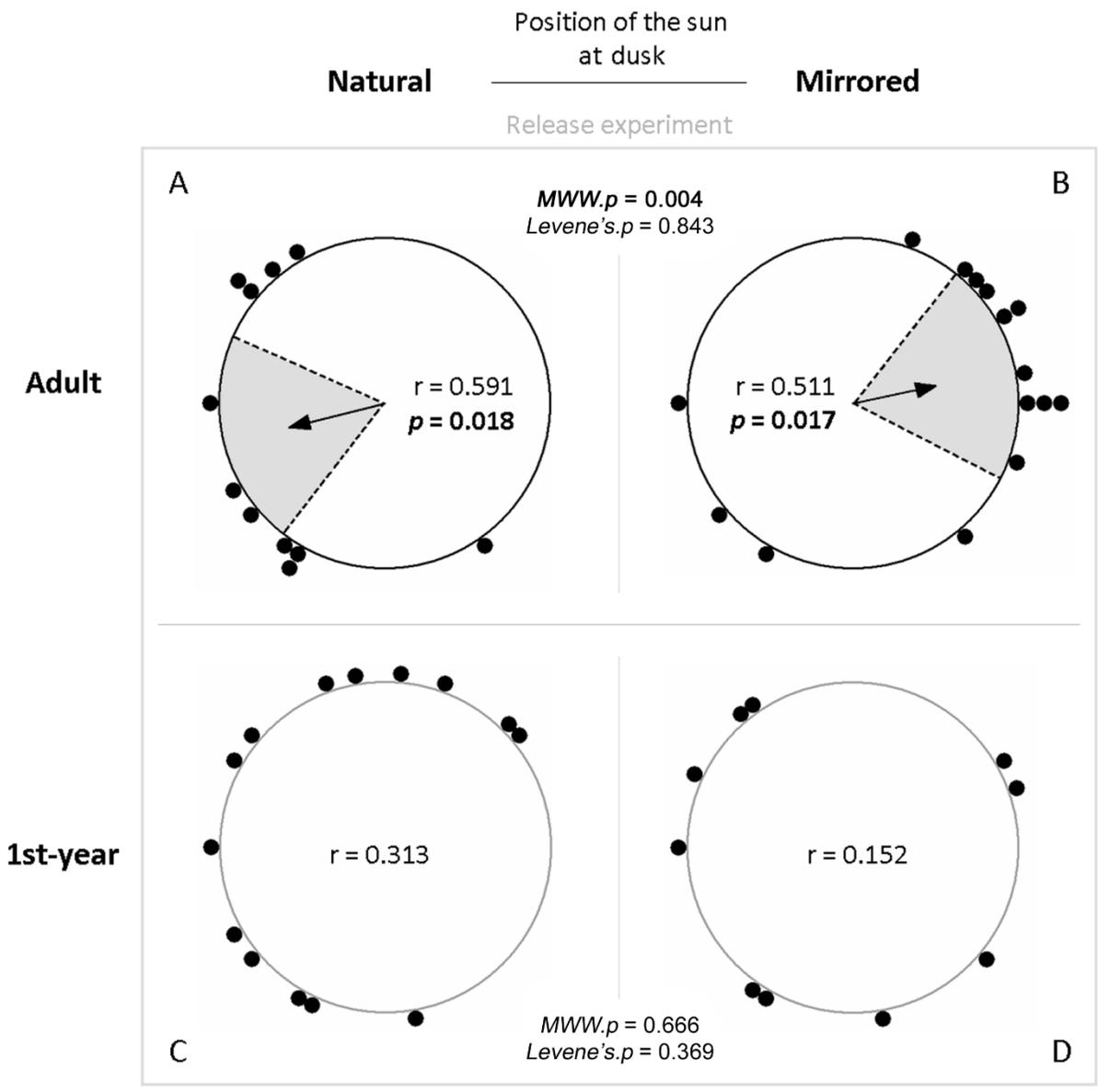
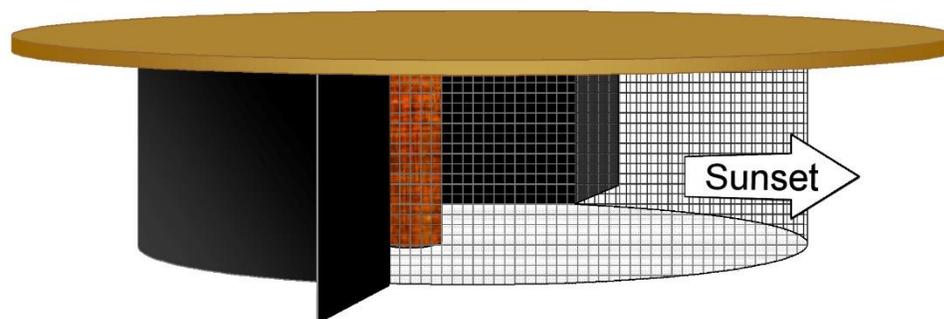


Figure 3.



STAR METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Soprano pipistrelle <i>Pipistrellus pygmaeus</i>	nature	N/A
Software and Algorithms		
Oriana (v4.0)	Kovach Computing Services	https://www.kovcomp.co.uk/oriana/index.html
Sigma Plot (v11.0)	Systat Software, Inc.	https://systatsoftware.com
Other		
Circular Release Box for Bats	self-made, see below for construction details and Figure 1.	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources should be directed to and will be fulfilled by the Lead contact, Oliver Lindecke (lindecke@izw-berlin.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental bats and site

Soprano pipistrelles (*Pipistrellus pygmaeus*) of both sexes were captured at Pape Bird Ringing Station (PBRS; 56°09' N 21°03' E, Rucava Municipality, Latvia) between 15 and 24 August 2017, using a Heligoland trap aligned to the Baltic Sea shore (dimensions at the entrance of the funnel 50 × 15 m). Soprano pipistrelle populations around the Baltic Sea move towards central

Europe in August and September [35, 36]. On the night of capture, bats were controlled for site-specific, seasonally appropriate body condition, aged according to the closure of the epiphyseal gaps of the phalanges, and afterwards transferred to a dark keeping room where wooden boxes served as cages for subsequent day-roosting. Bats were experimentally treated on the subsequent evening. The station and experimental sites are located in the Pape Nature Reserve. All work adheres to the ASAB guidelines for Use of Animals in Research and was carried out under the permission of the responsible Latvian authorities to the Institute of Biology, University of Latvia and Latvia University of Agriculture (Nr. 33/2017-E from 19.07.2017 and Nr. 3.6/85/2017-N-E issued by the Latvian Nature Conservation Agency), in accordance with the guidelines and regulations of the institutional animal care and ethics committee. The bats were released directly back into the wild when they were set free in the CRBox.

METHOD DETAILS

Mirror experiment

On the day of the experiment, any handling of test subjects was performed indoors in a wooden hut in order to avoid bats seeing the sun and any directional solar cues respectively, prior to the treatment phase. Experiments were only carried out during evenings with stable weather conditions such as zero wind to light breeze ($0\text{--}2\text{ m s}^{-2}$) and $0\text{--}60\%$ cloud cover and a visible sunset. The treatment site was 60 m from the research station, about 100 m from the shoreline and 150 m from the funnel trap. For transportation to the treatment site, bats were put singly into cotton bags (Ecotone, Gdynia, PL) and additionally into dark plastic buckets. For the experiment, bats were individually placed in cylindrical cages (20 cm diameter, 7 cm height; Figure 3) positioned on tables on the dune. For construction of these cages we chose materials which had no effects on the polarity or intensity of the local geomagnetic field, measured from inside the cage and compared with immediate surroundings of the treatment site using a conventional compass (Suunto A-30 NH Metric, Vantaa, FIN) and a smartphone (Apple iPhone 5) equipped with a Hall-Sensor (3D Magnetometer, Asahi Kasei Microdevices AK8963, Tokyo, JP) which was read out via *Phyphox* software (RWTH Aachen University, Aachen, GER). For this location, we measured the geomagnetic field intensity at $50.8\text{ }\mu\text{T}$ on 15 Aug 2017. The sidewall of a cage consisted of conventional gauze mesh (8 mm aperture) and was partly taped so that a bat inside would have a 180° panoramic view of the surroundings. The lid was made of cardboard and fitted with a wooden stick in the centre (2 cm diameter) oriented down to the bottom of the cage. Thus,

a bat could observe the environment from inside a cage in either horizontal body position or hanging heads down while clinging to the wood. We avoided using artificial light (flashlight or forehead lamps) for any procedure at the treatment site, so that the bats experienced natural sunlight, only. Further, according to the recently released atlas of artificial night sky brightness [37], our study site can be considered particularly dark at night, i.e., the natural nightscape is less polluted by anthropogenic light than the average European sky (ratio artificial to natural brightness < 0.05). The mirrors (stainless steel, 60×60 cm) were aligned perpendicular to the sunset bearing daily and positioned vertically, 50 cm in front of the caged bats. In general, when animals were handled, great care was taken that they neither saw the sky nor the true horizon until inserted into the cages and after completion of the treatment until they were put back in cotton bags to wait for translocation and release.

Circular release box assay

All components for the construction of the CRBox were purchased from hardware stores. The experimental setup consists of two circular shaped elements: an arena (42 cm diameter) as the bottom part and a lid (60 cm diameter; textured coated board) including the mechanism for remote release of the bat from a smaller acclimatisation box (8 cm diameter, 3 cm height; black plastic) in the centre (8 cm diameter) of the arena. The release mechanism is based on miniature electric motor (mounted onto the lid of the CRBox) which lifts the acclimatisation box for 3 cm when triggered remotely by radio control. For construction of the CRBox we only applied non-magnetic materials to avoid interference with a putative magnetic sense of bats. We made use of a flat funnel-shape for the arena (extruded polystyrol, XPS, Jackodur, Jackon Insulation), so that a bat starts a test below the level of the edge. The edge is 17 cm distant from the centre point of the CRBox. At the edge, the arena surface was again tilted downward 40° to facilitate takeoff for bats. The arena is coated with synthetic leather because the texture of this material supports crawling of bats and because it can be cleaned easily. A thin layer of chalk on the annulus around the acclimatisation box enables recording of the final takeoff direction based on tracks of a bat. The lid extends 9 cm beyond the arena, creating a brim that hinders the bat from seeing the night sky overhead and consequently impairs any celestial orientation at the RS. If the bat is motivated to gather information from the surroundings, it is thus forced to take off from the CRBox. For each trial the track of the previous test bat was eliminated with a renewed layer of chalk on the arena surface, and additionally, the CRBox was randomly rotated. Finally, the CRBox was

positioned 1 m above ground. The horizontal position of the apparatus was adjusted using a spirit level.

Geographical translocation and orientation testing

On the day of treatments after 2230 hours, we translocated the bats 5.1 km to the release site (RS) 98° east of the capture site away from the coastal migration corridor. By translocating the animals, we aimed to evade any bias on orientation behaviour which could arise from cues indicating the direction of the seashore, e.g. marine noise and visual landmarks provided by the dunes. The RS was on an open flat meadow (~3.1 ha) in the forested area of Pape Nature Reserve. Releases started at 2255 hours, the last trials during a given night were started between 0011 and 0239 hours. Before releasing bats individually, and during trials, we surveyed the vicinity of the RS for the presence of any other bats using a handheld ultrasound detector (D100, Pettersson Elektronik, Uppsala, SW). In the presence of other bats, experimental releases were paused to avoid confounding effects when released bats might have been biased in their vanishing direction. To measure the effect of our treatment on the bats' directional movements, we used a CRBox assay designed to record takeoff orientation on a full-circle. Previously, we identified takeoff orientation of as a useful proxy for departure flight direction of individually released adult bats [27]. Bats were randomly chosen for testing and were unable to explore the spatial details of the environment in which they could head prior to release from the CRBox. Therefore, we took care to keep bats naïve to the surroundings, i.e., we covered the individuals with cotton bags when they were carried to the CRBox. The direction for insertion of bats into the acclimatisation box was changed constantly between trials. The experimenter position relative to the CRBox was changed between trials in 90° steps, as well. We waited 15 sec before carefully lifting the acclimatisation box from 10 m distance using a remote control. The activity of the focal bat, i.e. its echolocation behaviour, was constantly monitored using a bat detector. Based on a pilot study in adult pipistrelles, we set a threshold time of 3 min until a trial was cancelled in case no echolocation indicated a lack of movement activity [27]. One bat took longer than this time threshold and consequently was ignored in the analysis. Free flight after takeoff was indicated by a sequence of echolocation calls leading away from the CRBox. However, we misinterpreted echolocation calls in three cases and exposed these individuals to light prior to takeoff. We excluded these trials. If a takeoff could be clearly identified by means of echolocation calls, we noted the time (sec), i.e. the takeoff latency a focal bat took to depart

from the CRBox after the acclimatisation box was lifted. Takeoff latency potentially indicates behavioural differences between test groups [27]. We recorded takeoff latencies in 37 trials (C=18; M=19). After a bat had disappeared from the RS, we measured the bearing for takeoff, i.e. takeoff orientation, using a compass which was placed in the centre of the CRBox. The experimenter who recorded orientation data was blind to the treatment of bats. Over the course of nine nights, we recorded takeoff orientations of 26 adult (10 male, 16 female) and 24 subadult (11 male, 11 female, 2 sex undetermined) bats.

QUANTIFICATION AND STATISTICAL ANALYSIS

Takeoff bearings were analysed using the Oriana 4.0 circular statistics software package (Kovach Computing Services, Pentraeth, UK). We calculated mean bearings and vector lengths, and then Kuiper's omnibus test was used for each treatment group as a goodness-of-fit test against uniform circular distribution, i.e., to detect randomness of data [38]. If Kuiper's test statistic indicated non-random orientation of data, the Rayleigh test was used to test for unimodal departure from circular uniformity, i.e. a preferred takeoff orientation [39]. We compared variances in group-specific orientation scatter by applying the Levene's test based on the deviations of individual takeoff bearings from the group-specific mean [40]. The Mardia–Watson–Wheeler test was selected to test for differences in distribution across groups [38]. Testing for differences in takeoff latency was performed using t-tests for adult and subadult groups, and the treatments, respectively (SigmaPlot 11.0, Systat Software Inc., Chicago, USA).

DATA AND SOFTWARE AVAILABILITY

The heading data used in the statistical analysis for this study and a map showing the study location and mean orientations of adult test groups can be found in the supplementary materials associated with this article, available online (Data S1 file and Figure S2).