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Orientation of lizards in a Morris water-maze: roles of the sun compass and the parietal eye

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SUMMARY

The present study examined for the first time whether a Morris water-maze can be used to explore compass and other orientation mechanisms in the ruin lizard *Podarcis sicula*. In the open field, during sunny days, lizards were individually trained to swim from the center of the water maze onto a hidden platform (the goal), positioned at the periphery of the maze in a single compass direction. The goal was invisible because it was placed just beneath the water surface and the water was rendered opaque. The results showed that lizards learn to swim directly towards the hidden goal under the sun in the absence of visual feature cues. We further examined whether the observed orientation response would be due to lizards learning the spatial position of the goal relative to the sun's azimuth, i.e. to the use of a time-compensated sun compass. Lizards reaching learning criteria were subjected to 6 h clock-shift (fast or slow), and tested for goal orientation in the Morris water-maze. Results demonstrated that the learned orientation response is mediated by a time-compensated sun compass. Further investigations provided direct evidence that in ruin lizards an intact parietal eye is required to perform goal orientation under the sun inside a Morris water-maze, and that other brain photoreceptors, like the pineal or deep brain photoreceptors, are not involved in orientation.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/18/2918/DC1

Key words: lizard orientation, Morris water-maze, sun compass, parietal eye.

INTRODUCTION

Although a homing ability from sites outside their home range was documented in several species of lizards, the underlying orientation mechanisms are relatively unknown in these reptiles (Freake, 2001). Some investigations showed that lizards can use a time-compensated sun compass: orientation of 6 h fast or slow clock-shifted spiny lizards (*Sceloporus jarrovi*) deviates in the expected directions either in homing experiments in the field (Ellis-Quinn and Simon, 1991) or in orientation tests performed in an outdoor arena after directional training [fringe-toed lizard *Uma notata* (Adler and Phillips 1985)]. Homing experiments suggested that sleepy lizards (*Tiliqua rugosa*) use celestial cues to determine the compass bearing of the outward journey and reverse this bearing to orient in the homeward direction [course reversal (Freake, 2001)]. Further field work in *S. jarrovi* and *T. rugosa* examined whether the parietal eye of lizards is involved in homing orientation: lizards of both species orientated at random when the parietal eye was entirely covered with a patch whereas control individuals fitted with a sham parietal eye patch were homeward orientated (Ellis-Quinn and Simon, 1991; Freake, 2001). Interestingly, the lizard parietal eye was proposed to mediate a sky polarization compass sense but no experiments were carried out so far to directly test that hypothesis (Adler and Phillips, 1985; Ellis-Quinn and Simon, 1991; Freake, 1999; Freake, 2001).

Previous investigations in rats showed that the technique of using escape from water inside a Morris water-maze can be very successful to motivate spatial learning (Morris, 1984). Based on field observations individual lizards (*Podarcis sicula* and *Podarcis muralis*) were often reported to swim across small water streams, suggesting that swimming behavior is neither unnatural nor unusual in Lacertidae (A.F., personal observations).

In the present paper we examined for the first time whether a Morris water-maze can be used to explore compass and other orientation mechanisms in the ruin lizard *P. sicula*. For this purpose, ruin lizards were individually trained to swim from the center of the water maze onto a hidden platform (the goal), positioned at the periphery of the maze in a given compass direction. Experiments were performed outdoors, during sunny days, in the absence of visual feature cues. As the present results showed that ruin lizards can learn to swim directly towards the hidden goal under the sun with no visual feature cues, we decided to examine whether the orientation response would be due to lizards learning the spatial position of the goal relative to the sun's azimuth, i.e. to the use of a time-compensated sun compass. For this purpose, lizards reaching learning criteria were subjected to 6 h clock-shift (fast or slow) and then tested for orientation in the Morris water-maze. Further investigations tested whether an intact parietal eye is necessary for goal orientation in the Morris water-maze under the sun. To distinguish between the role of the parietal eye and the possible role of other photoreceptive structures contained in the brain [pineal and deep brain photoreceptors (Pasqualetti et al., 2003; Bertolucci and Foà, 2004)], we compared the orientation of lizards whose parietal eyes were black-painted with the orientation of lizards with ablated parietal eyes.

MATERIALS AND METHODS

Animals

Ruin lizards (*Podarcis sicula* Rafinesque-Schmaltz 1810; adult males only, 6.5–8 cm snout–vent length) were collected by means of traps baited with mealworms (*Tenebrio molitor* larvae) from the area of Ferrara (Italy; longitude: 12°21′44″E, latitude: 45°03′72″N) under the authority of the Parco Delta del Po-Emilia Romagna (Department of Wildlife and Fisheries). After capture lizards were immediately transported to the lab in tissue bags and placed in vivaria (100×55×52 cm high), with about 5–6 cm of sand on the bottom, and some air bricks for cover. Vivaria were exposed to the natural photoperiodic conditions. A heat lamp (Siccatherm 250 W, Osram GmbH, Munich, Germany) was placed 60–70 cm above the ground on one side of each vivarium to allow lizards to thermoregulate. Substrate temperatures varied from 36°C directly below the lamp to 22°C at the far end of the vivarium. Heat lamps were on from 09:00 h to 17:00 h every day. The day before starting with pre-training in the Morris water-maze lizards were painted on their backs with non-toxic colors (red, yellow, blue, orange, white and black; Craft color, Bologna, Italy) for individual marking, placed in groups of three–four individuals inside plastic containers (43×15×26 cm high) and then moved to environmental chambers kept at constant temperature (29±0.5°C) and exposed to a light–dark cycle corresponding to the natural photoperiod. Visual observations indicated that color marking did not affect the behavior of the lizards. In the chambers, lighting was provided by cool daylight fluorescent tubes (FH HE/860 Lumilux T5, Osram GmbH), giving an intensity of 200 lx at the level of the lizard head. The light intensity was measured by a photometer (PCE172, PCE Group, Lucca, Italy). Food (*T. molitor* larvae) was supplied twice a week, and water was available *ad libitum* both in the vivaria and in the chambers. General animal care was carried out by veterinary and professional staff not associated with the research team. The captive maintenance procedures and research protocols were approved by the University of Ferrara Institutional Animal Care and Use Committee and by the Italian Ministry of Health. At the end of experiments lizards were released in the area in which they were captured. At that time marking had completely faded.

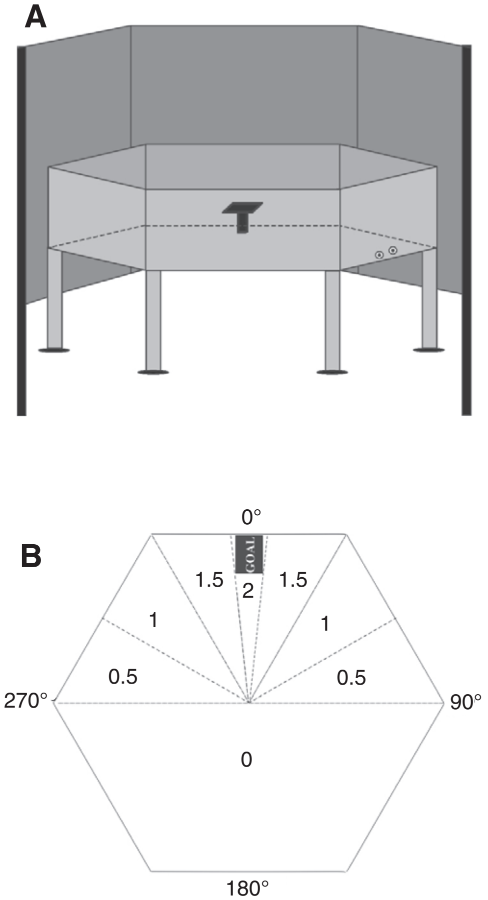

Fig. 1. (A) Schematic drawing of the hexagonal Morris water-maze used in experiments. Figure shows the poles raising the maze from the ground and the goal platform mounted upon its pedestal located at the periphery of the maze in the center of a side wall. The fence completely surrounding the water maze to prevent lizards from seeing the landscape is partially shown. Further details are reported in the Materials and methods section. (B) Scheme of the water maze surface reporting the score system used to establish learning criteria. The top of the hexagon identifies the goal platform (direction 0°).

Experimental apparatus

Instead of circular, as was the water maze proposed by Morris (Morris, 1984), the present testing apparatus (Fig. 1A) was a hexagonal maze (diagonal 182 cm, apothem 77 cm, side walls 93 cm long and 40 cm high) made of opaque (gray) polyvinyl chloride (PVC) and mounted upon PVC poles raising the bottom of the maze 82 cm high from the ground. The maze was filled with water to a depth of 15±0.5 cm. Water was maintained at a constant temperature of 29±1.0°C by means of aquarium thermostats (Tetraht, Tetra, Milano, Italy) and rendered opaque by the addition of fossil flour (Clarcel, Ceca, Honfleur, France). The goal was a Plexiglas, transparent, rectangular platform (23.7×16×2.5 cm thick), mounted upon a pedestal (11.5 cm high from the maze bottom). The goal was positioned in direct contact with the center of a side wall, in a given compass direction. The maze was placed outdoors and was surrounded by a fence made of green plastic to a height of 220 cm (Fig. 1A). This prevented the lizards from seeing landscape features, without obstructing the view of the sky overhead. Peepholes were built through the green plastic of the fence, allowing observers to follow lizard behavior during releases and to place the lens of a movie camera (GZ-MG21E, JVC, Japan), which recorded all lizard trials.

Maintenance of animals outdoors

One hour before starting with the individual releases, lizards were transferred from the environmental chambers to glass containers placed outdoors in shaded areas, near to the Morris water-maze. Glass containers (60×24×36 cm high) were filled with 10 cm of water which was maintained at 29±1.0°C constant temperature by means of aquarium thermostats. A wire net placed 0.5 cm under the water surface allowed lizards to remain wet without sinking. In this way lizards in the containers were familiarized with the water environment before release and also kept at the same temperature of the water of the maze.

Release conditions and procedures

During experiments the sun disc was always visible. Experiments were carried out between 11:00 h and 14:00 h from June to September 2007.

Release box

For release a lizard was taken from the container and placed inside a black release box (21×8×4 cm high), which was covered with a black lid. The release box was placed in the water, after which the experimenter took off the lid and moved immediately out of the sight of the lizard. A small (125 g) brass anchor connected with a nylon string to the bottom of the release box prevented the box from floating away from the starting release position. Due to many small (4.5 mm) holes in its floor, the release box sunk within 6–7 s, so that the lizard was forced to swim.

Common procedures

For each lizard the compass direction of the goal always remained the same whereas the release order changed in a random sequence from one trial series to another, both during pre-training and training.

Pre-training

The goal platform was above the opaque water surface. In the first trial the release box containing the lizard was placed in direct contact with the goal. In the subsequent three trials, the release box was moved away, progressively approaching the center of the Morris water-maze. Once at the center, the release box was faced in one of four directions, assigned randomly in four subsequent trials.

Training

For training, the surface of the goal platform was placed just beneath the opaque water surface and was thus invisible to the lizards (see Movie 1 in supplementary material). Each lizard was released from the center of the maze, by orienting the release box each time in a different direction. The straight course from the center of the maze to the side wall containing the goal measured 77 cm. The first point of a side wall touched by a lizard was recorded as the directional choice in that trial. After each trial the side walls of the maze were cleaned to avoid the appearance of unevenness in the visual field of lizards, which might be used as orienting feature cues. Lizards reaching the goal or at least the side wall containing the goal were rewarded, and their trials were scored 2 and 1.5, respectively (Fig. 1B). For a lizard getting to the goal platform, the reward consisted of immediately lowering the water level in the maze, so that the goal and the lizard placed on it could emerge completely from water within 5–6 s. The lizard was kept there for 30 s before recapture. In case a lizard reached the correct side wall, but not the goal platform, the water level was lowered as described above and then the lizard was gently placed upon the emerged goal for 30 s as a reward. Lizards reaching one of the two side walls contiguous to the goal side wall were not rewarded, and their trials were scored from 1 to 0.5, with a lower score further from the goal (Fig. 1B). Trials of lizards swimming towards one of the remaining three side walls were considered incorrect, and their trials were scored 0 (Fig. 1B). In any case, lizards reaching one of the five side walls not including the goal were left in the water for 30 s before recapture. Sometimes lizards sank immediately at release and after 30–65 s spent underwater re-emerged and swam around in circles or, more often, escaped towards the nearest wall of the maze. In all of these situations lizard directional choices were discarded and the trial of the unsuccessful lizard was repeated. Successful trials were grouped in training sessions, each made up of six trials.

Measurement of lizard's compass bearings

In each trial, the compass direction of the first point of a side wall touched by each lizard was measured from the center of the maze by means of an azimuth compass (Wayfinder Outback ES, Sphere Innovative Technologies, Kingsford, NSW, Australia). This compass direction was recorded as the directional choice of the lizard in the current trial.

Learning criteria

To reach learning criteria each lizard had to be awarded a score of six or higher within each of two consecutive training sessions, with a maximum of 1 trial scoring ≤0.5 (Fig. 1B).

Pilot experiment to establish learning capability and retention

Fifteen lizards were given a pre-training session of eight trials (two trials per day) and three training sessions for a total of 18 trials (two trials per day). After the end of the last training session lizards were kept in environmental chambers for seven days. At the end of this period of time lizards reaching criteria were subjected to a final orientation test to establish whether they would recall the direction of the goal after seven days with no trials. Such a retention test was carried out in the absence of the goal platform (no reward was available during testing). Initially, the retention test of lizards reaching criteria was planned to consist of a six trials–test session to obtain orientation data comparable with those of the last six trials–training session. This was, however, unfeasible, because already by the third trial of the test session several lizards swam around in circles, apparently looking for the missing goal platform, without making a directional choice. Thus, only the first trial test was considered valid, and only the orientation data of the last (sixth) trial of the last training session were used for comparison. Lizards that failed to reach criteria were subjected to another (fourth) training session.

Clock-shift tests

A new group of lizards (*N*=45) underwent a pre-training session (eight trials, two trials per day), and two–three training sessions (12–18 trials, two trials per day) until they reached learning criteria. Lizards (*N*=23) failing to reach criteria within three training sessions were excluded from experiments. After reaching criteria, each lizard was attributed to one of the three following treatments: (1) housing for seven days in an environmental chamber where the artificial day started and ended in coincidence with the natural light phase (control, *N*=8); (2) housing for seven days in an environmental chamber where the artificial day started and ended 6 h after the natural light phase (slow clock-shift, *N*=7); and (3) housing for seven days in an environmental chamber where the artificial day started and ended 6 h before the natural light phase (fast clock-shift, *N*=7). After completion of the clock-shift treatment lizards were tested to determine if they had learned the goal location by means of their sun compass. For each clock-shifted lizard the orientation test consisted of one trial in the absence of the goal platform. For each group of lizards – fast clock-shift, slow clock-shift and control – the orientation data of the last trial of the last training session were used for comparison.

Parietal eye painted test (PAR-P)

A new group of lizards (*N*=28) underwent a pre-training session (eight trials, two trials per day), and two–three training sessions (12–18 trials, two trials per day) until they reached learning criteria. Lizards (*N*=15) failing to reach criteria within three training sessions were excluded from experiments. After reaching criteria, each lizard was brought back to the environmental chambers until the next day, when it was attributed to one of the two following treatments: (1) the parietal eye (supplementary material Fig. S1A) was painted black (PAR-P, *N*=7) (supplementary material Fig. S1B); and (2) a scale alongside the parietal scale was painted black (SHAM PAR-P, *N*=6) (supplementary material Fig. S1C). The non-toxic color (Craft color) was painted by means of a fine paintbrush 1 h before the orientation test. The orientation test consisted of one trial in the absence of the goal platform for each PAR-P and SHAM PAR-P lizard. For each lizard of both groups the orientation data of the last trial of the last training session were used for comparison.

Parietal eye ablated test (PAR-X)

At the end of the orientation test each SHAM PAR-P lizard was subjected to ablation of the parietal eye, becoming in that way a PAR-X lizard (*N*=6) (supplementary material Fig. S1D). Surgical procedures were similar as previously reported (Innocenti et al., 1993). Briefly, SHAM PAR-P lizards were first cooled in a refrigerator (1–4°C) until immobilized and then embedded in crushed ice. A small lancet was used to scrape away the black spot of paint covering the scale alongside the parietal scale. PAR-X was performed by scraping away the parietal eye by means of a microcurette (diameter 0.5 mm; FST, Vancouver, Canada), after which the parietal eye foramen could be perfectly visualized underneath the eye (supplementary material Fig. S1D). Surgery lasted 5–10 min, after which PAR-X lizards were warmed up under an incandescent bulb (100 W, Osram GmbH) and then brought back to their plastic containers. The orientation test took place the day after surgery and consisted of one trial in the absence of the goal platform for each PAR-X lizard.

Data analysis and statistics

In all experiments the orientation data were normalized across subjects by assigning a direction of 0° to the midpoint of the goal platform.

Pilot experiment

The directional choices of each lizard over the six trials of each training session were used to calculate an individual mean vector per session (Batschelet, 1981). In each training session the *V*-test was used to test whether the directional choices of each lizard deviated from uniform (Batschelet, 1981). For each training session the individual mean vectors were used to calculate a session mean vector. The Hotelling test was used to test whether the directions of the individual mean vectors of each session deviated from uniform. For lizards reaching criteria, the Hotelling test for paired data was applied to test for differences between the directional choices these animals showed in the last trial of the last training session and the directional choices they expressed after seven days with no training (retention test). We calculated the scores awarded to each lizard across the six trials of each training session. A one-way analysis of variance (ANOVA) for repeated measures was applied to these scores to test for scores differences among sessions.

Clock-shift and parietal eye tests

For each treatment, the directional choices expressed by lizards during the one trial orientation test were used to calculate the group mean vector, and the *V*-test was used to test whether directional choices deviated from uniform (Batschelet, 1981). For each treatment, the Hotelling test for paired data was applied to test for differences between the directions chosen by lizards in the last trial of the last training session and the directions chosen by the same lizards in the one trial orientation test (Batschelet, 1981). Comparisons between different treatments were made using the Watson U^2 test (Batschelet, 1981).

RESULTS

Pilot experiment

Eight out of 15 lizards reached criteria. Their directional choices are shown in Fig. 2A–C. Taken as a group, the directional choices of lizards that reached criteria within the three training sessions were not orientated in session one (Hotelling test: $F_{2,6}$=1.02, P>0.25; group mean vector direction: 347°; group mean vector length: 0.2) but were orientated both in session two (Hotelling test: $F_{2,6}$=81.16, P<0.0005; group mean vector direction: 338°; group mean vector length: 0.7) and in session three (Hotelling test: $F_{2,6}$=102.23, P<0.0005; group mean vector direction: 348°; group mean vector length: 0.7). Scores awarded in session three were significantly higher than scores awarded in session one (one-way ANOVA for repeated measures: $F_{2,14}$=7.03, P<0.01), showing an overall improvement of performances from session one to session three. The fourth session of training that was reserved to lizards which did not reach criteria within three sessions was unsuccessful (data not shown). Fig. 3A,B reports the results of the retention test. In the last trial of the last training session lizards were orientated (Fig. 3A) (*V*-test: u=3.22, P<0.0005; group mean vector direction: 336°; group mean vector length: 0.9). After seven days with no training (Fig. 3B), lizards were still orientated (*V*-test: u=2.37, P<0.01; group mean vector direction: 329°; group mean vector length: 0.7), and their directional choices did not differ from those expressed in the last trial of the last training session (Hotelling test for paired data: $F_{2,6}$=0.87, P>0.25). The fact that lizards reaching criteria were still orientated toward the goal after seven days with no training suggests the existence of good retention of the learned spatial task.

Clock-shift tests

The directions chosen by lizards, respectively, attributed to control (N=8) (Fig. 4A), slow clock-shift (N=7) (Fig. 4B) and fast clock-shift (N=7) (Fig. 4C) in the last trial of the last training session deviated from uniform (control: *V*-test: u=2.58, P<0.005; group mean vector direction: 8°; group mean vector length: 0.65; slow

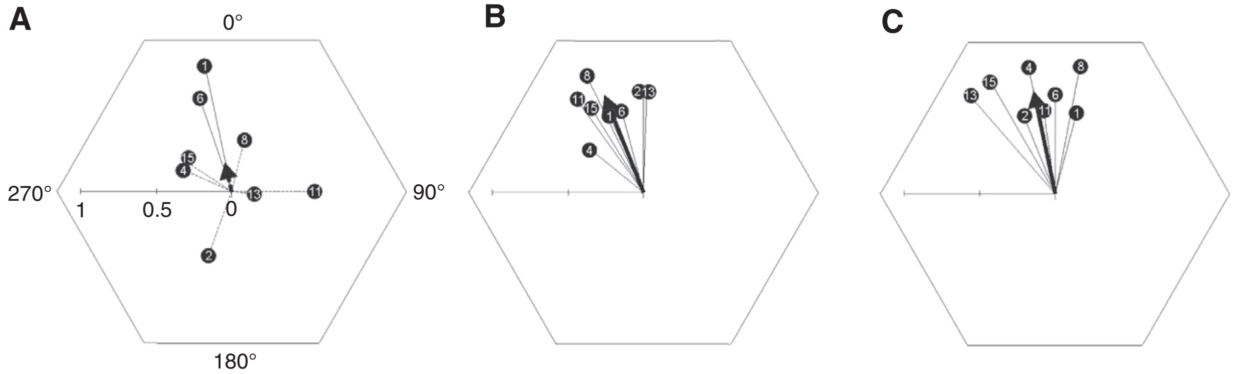

Fig. 2. (A–C) Pilot experiment. Orientation performances of lizards reaching learning criteria within three training sessions. Lines inside each hexagon represent individual mean vectors, the length of which can be read from the scale. Each individual mean vector is calculated on the six directional choices of one lizard in one session. The number at the top of each mean vector identifies the lizard. Each dotted line indicates that the directional choices of a lizard in a training session do not deviate from uniform (*V*-test). A, B and C refer to training sessions one, two and three, respectively. For each training session the individual mean vectors were used to calculate a session mean vector, represented by a solid arrow. The dotted mean vector in A indicates a random distribution of individual mean vectors (Hotelling test). The goal direction is 0°. Note that the number at the top of each individual mean vector identifies the same lizard in Figs 2 and 3.

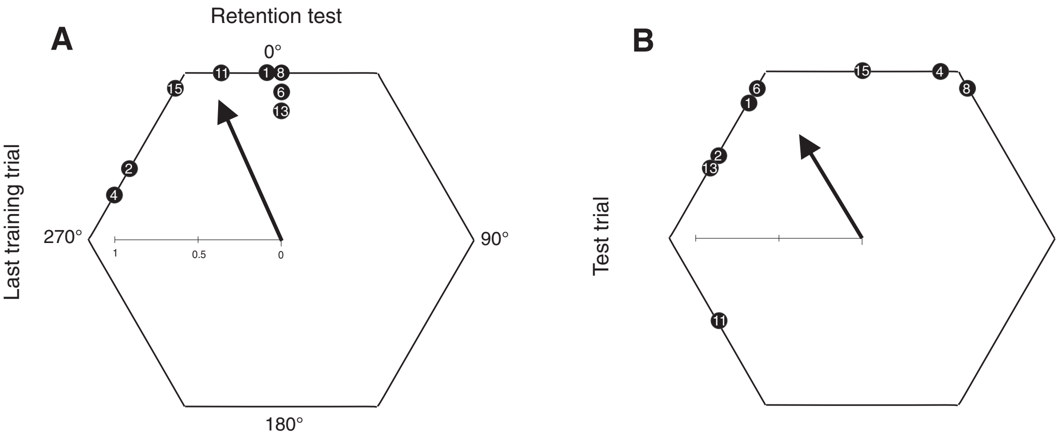

Fig. 3. (A,B) Pilot experiment. Directional choices of lizards in the last trial of the last training session (A) and in the retention test carried out after seven days with no training (B). Each symbol indicates the directional choice of a single lizard identified by its number. In each hexagon the inner arrow represents the mean vector of the group, the length of which can be read from the scale. The goal direction is 0°.

clock-shift: *V*-test: u=2.64, P<0.005; group mean vector direction: 358°; group mean vector length: 0.7; fast clock-shift: *V*-test: u=3.42, P<0.0005; group mean vector direction: 349°; group mean vector length: 0.9). In the orientation test the directional choices of the control group deviated from uniform (*V*-test: u=2.92, P<0.001; group mean vector direction: 352°; group mean vector length: 0.85) (Fig. 4D), and did not differ from those expressed by the same lizards in the last trial of the last training session (Fig. 4A) (Hotelling test for paired data: $F_{2,6}$=0.75, P>0.25). In the orientation test after clock-shift treatment the slow clock-shift lizards were still orientated (Fig. 4E), and their group mean vector deviated clockwise with respect to the goal (expected direction: 90°, *V*-test: u=1.94, P<0.05; group mean vector direction: 47°; group mean vector length: 0.7). The directions chosen by the slow clock-shift lizards in the orientation test were significantly different from those of the same lizards in the last training trial (Fig. 4B) (Hotelling test for paired data: $F_{2,5}$=8.44, P<0.025). In the orientation test after clock-shift treatment the fast clock-shift lizards were still orientated (Fig. 4F), and their group mean vector deviated counter-clockwise with respect to the goal (expected direction: 270°, *V*-test: u=2.59,

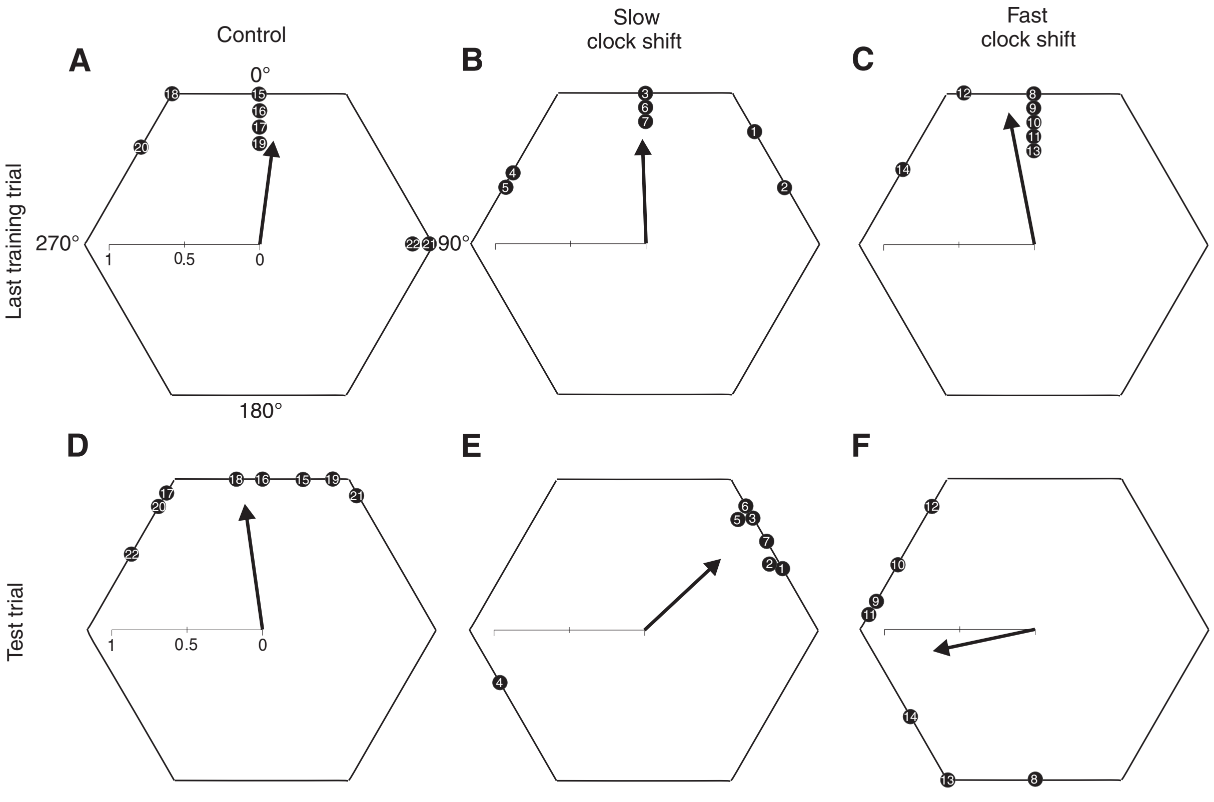

Fig. 4. (A–F) Clock-shift tests. Control lizards: directional choices in the last trial of the last training session (A) and in the single orientation test carried out seven days later (D). Slow clock-shift lizards: directional choices in the last trial of the last training session (B) and in the single orientation test carried out after the 6 h slow clock-shift (E). Fast clock-shift lizards: directional choices in the last trial of the last training session (C) and in the single orientation test carried out after the 6 h fast clock-shift (F). Further information in Fig. 3.

$P<0.005$; group mean vector direction: 258°; group mean vector length: 0.7). The directions chosen by the fast clock-shift lizards in the orientation test were significantly different from those of the same lizards in the last training trial (Fig. 3C) (Hotelling test for paired data: $F_{2,5}=15.81$, $P<0.01$). Furthermore, the directions chosen by slow clock-shift lizards were significantly different from those of fast clock-shift (Watson U^2 test: $U^2_{7,7}=0.20$, $P<0.05$) and control lizards (Watson U^2 test: $U^2_{8,7}=0.32$, $P<0.005$).

Parietal eye painted test

The directions chosen by lizards, respectively, attributed to PAR-P ($N=7$) (Fig. 5A) or SHAM PAR-P group ($N=6$) (Fig. 5B) in the last trial of the last training session deviated from uniform (PAR-P: V-test: $u=2.38$, $P<0.01$; group mean vector direction: 330°; group mean vector length: 0.7; SHAM PAR-P: V-test: $u=3.11$, $P<0.0005$; group mean vector direction: 11°; group mean vector length: 0.9). In the orientation test the directional choices of the SHAM PAR-P group deviated from uniform (V-test: $u=2.69$, $P<0.005$; group mean vector direction: 354°; group mean vector length: 0.8) (Fig. 5D) whereas those of the PAR-P group did not (V-test: $u=0.13$, $P>0.25$; group mean vector direction: 194°; group mean vector length: 0.04) (Fig. 5C).

Parietal eye ablated test

The directional choices of PAR-X lizards ($N=6$) (Fig. 5E) in their orientation test did not deviate from uniform (V-test: $u=-0.02$, $P>0.25$; group mean vector direction: 269°; group mean vector length: 0.3).

DISCUSSION

The present study showed for the first time that a Morris water-maze can be used to explore compass orientation mechanisms of lizards. Outdoors, with the sun disc visible, ruin lizards *P. sicula* were requested to swim from the center of a Morris water-maze onto a hidden goal, positioned at the periphery of the maze in a given compass direction (Movie 1 in supplementary material). Our results actually showed that ruin lizards have learned the spatial position of the hidden goal at the periphery of the Morris water-maze (Figs 2 and 3). The fact that experiments were always carried out under the sun and in the absence of visual feature cues supports the view that the observed orientation response could have been mediated by a time-compensated sun compass. To test this possibility, ruin lizards reaching criteria were subjected to a 6 h clock-shift, either fast or slow. The results showed that the directional choices of both experimental groups deviated in the expected directions. In fact the mean vector of slow clock-shift lizards deviated clockwise and that of fast clock-shift lizards deviated counter-clockwise with respect to the goal (Fig. 4). However, while the deviation of fast clock-shift lizards was as large as expected for a 6 h clock-shift (102° deviation with respect to the goal, 94° with respect to control lizards), the deviation of slow clock-shift lizards was reduced in amplitude (47° deviation with respect to the goal, 55° with respect to control lizards). Deviations of reduced amplitude after a 6 h clock-shift were reported in the directional choices of birds and were interpreted as the result of a compromise between the use of a (shifted) sun compass and that of the available visual cues (Sherry and Duff, 1996; Duff et al., 1998; Gagliardo et al.,

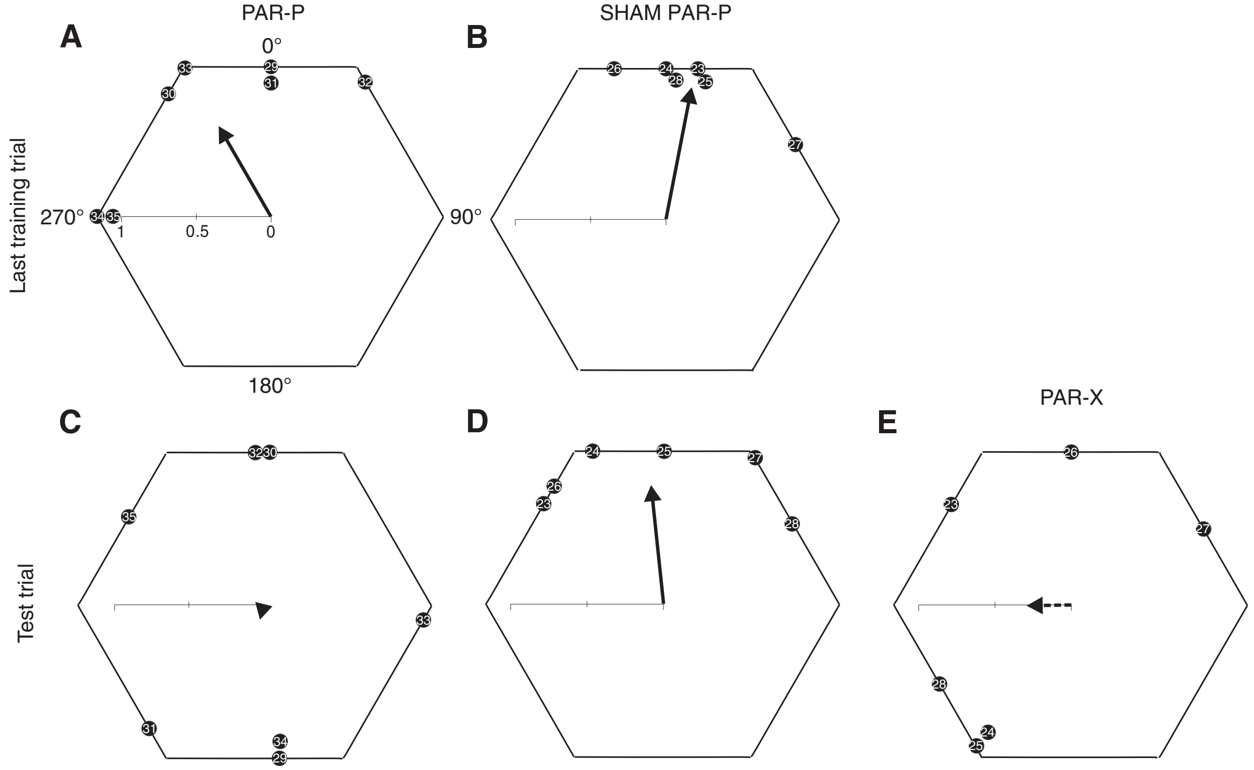

Fig. 5. (A–E) Parietal eye tests. PAR-P lizards: directional choices in the last trial of the last training session (A) and in the orientation test carried out after the parietal eye had been painted black (C). SHAM PAR-P lizards: directional choices in the last trial of the last training session (B) and in the single orientation test carried out after a scale alongside the parietal scale had been painted black (D). After the orientation test, SHAM PAR-P lizards were subjected to ablation of the parietal eye (PAR-X). The results of the single orientation test carried out on PAR-X lizards are shown in E. Further information in Fig. 3.

2005). This is, however, highly unlikely in the present experiments for the following reasons. (1) The water-maze was surrounded by a 220 cm high solid fence, which prevented ruin lizards from seeing landscape. (2) The side walls of the maze were cleaned after each trial to avoid lizard detection of unevenness that might be used as orienting visual cues. Furthermore, and perhaps more relevant, (3) the amplitude of the deviations of fast clock-shift lizards, which were tested during the same days and at the same times of day as slow clock-shift lizards, were not reduced. Interestingly, Iosalè et al. (Ioalè et al., 2000) reported in homing pigeons that fast clock-shift can be more effective than slow clock-shift, although the reasons for such a discrepancy are at present unknown. Overall, based on our clock-shift data, it seems difficult to escape the conclusion that the orientation response of ruin lizards in the Morris water-maze is due to learning of the spatial position of the goal relative to the sun's azimuth, by using a time-compensated sun compass.

Further experiments examined whether sun-compass-mediated goal orientation inside the Morris water-maze requires the presence of an intact parietal eye. Because painting the parietal eye might prevent from perceiving light not only the parietal eye itself but also the pineal and deep brain photoreceptors (Bertolucci and Foà, 2004), we compared the directional choices of lizards whose parietal eyes were painted black (PAR-P) with the directional choices of lizards whose parietal eyes were completely ablated (PAR-X) (Fig. 5D,E). The results showed that the directional choices of either PAR-P or PAR-X lizards were randomly orientated inside the Morris water-maze. This supports the view that only the parietal eye – and not other brain photoreceptors – plays a role in lizard orientation. Overall, the present data show that in ruin lizards an intact parietal eye is required to perform goal orientation under the sun inside a Morris water-maze. This confirms the results of field investigations carried out in the spiny lizard *S. jarrovi* and the sleepy lizard *T. rugosa*, which already indicated a crucial role of the parietal eye in lizard orientation under the sun (Ellis-Quinn and Simon, 1991; Freake, 2001). In both experiments lizards released in sunny days outside their home ranges were incapable of orientating homeward when their parietal eyes were covered with a patch (*T. rugosa*) or painted black (*S. jarrovi*). Furthermore, the data of either Ellis-Quinn and Simon (Ellis-Quinn and Simon, 1991) or Freake (Freake, 2001) are conclusive in showing that the disorientation observed after covering the parietal eye is due to a direct effect on an orientation mechanism and not to unspecific effects, such as reduced motivation or altered thermoregulation. Because in all of these experiments (including the present one) lizards with fully covered parietal eyes were disoriented in spite of the fact that their lateral eyes were unobstructed, the data so far available strongly suggest that contribution of the lateral eyes to sun-compass orientation of lizards is apparently irrelevant. Interestingly, several authors proposed that the lizard parietal eye could mediate a sky polarization compass sense, which may provide an advantage over a sun azimuth compass, because it remains functional even when the sun's position is obscured by vegetation and clouds (Adler and Phillips, 1985; Ellis-Quinn and Simon, 1991; Freake, 1999; Freake, 2001). This view is supported so far by anatomical data suggesting that the photoreceptors in the parietal eye are arranged in a pattern that would theoretically allow for analysis of the e-vector of linearly polarized light (Hamasaki and Eder, 1977; Freake, 1999). Results of experiments carried out by Adler and Phillips (Adler and Phillips, 1985) in the fringe-toed lizard *U. notata* and by Freake (Freake, 1999) in the sleepy lizard *T. rugosa* support the hypothesis that lizards can use the e-vector direction of polarized light in the form of a sky polarization compass; however, the effective role of the parietal eye has not been examined so far.

On the whole, the present results established that a Morris water-maze can be useful to study lizard orientation mechanisms. The fact that two–three relatively short training sessions (six trials each) were enough for ruin lizards to reach criteria suggests that escape from water is quite an efficient technique to motivate learning of the spatial position of the goal. In this respect, our results confirm those of previous investigations in rats, showing that escape from water can be used successfully to motivate spatial learning (Morris, 1984). However, unlike rats, because lizards are ectothermic animals, it is important to point out here that maintenance of temperature levels suitable for activity in each phase of the experiments has been crucial to get meaningful results. We kept environmental chambers as well as the water of the maze at a temperature of 29±1.0°C, because it is comprised within the range of body temperatures best suited for activity in ruin lizards (Licht, 1969; Tosini et al., 1992). Ambient temperatures of 29±1.0°C were also used successfully to monitor locomotor activity of ruin lizards in previous laboratory experiments (Foà, 1991; Foà and Bertolucci, 2001). That such temperature levels were well suited also for water experiments is shown by the fact that ruin lizards were clearly capable of making directional choices inside our Morris water-maze.

We have recently planned to further explore compass orientation mechanisms of ruin lizards in a Morris water-maze, and the next step of investigation will be aimed at answering the still open question of whether functioning of the putative sky polarization compass of lizards is effectively mediated by the parietal eye.

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