

## Exposure to hypomagnetic field space for multiple generations causes amnesia in *Drosophila melanogaster*

Bin Zhang<sup>a,c</sup>, Huimin Lu<sup>a</sup>, Wang Xi<sup>b</sup>, Xianju Zhou<sup>b</sup>,  
Shiyu Xu<sup>a</sup>, Ke Zhang<sup>b</sup>, Jinchang Jiang<sup>a</sup>, Yan Li<sup>a</sup>, Aike Guo<sup>a,b,\*</sup>

<sup>a</sup> Laboratory of Visual Information Processing, Center for Brain and Cognitive Sciences, Institute of Biophysics, CAS, 15 Datum Road, Chaoyang District, Beijing 100101, China

<sup>b</sup> Institute of Neuroscience, Shanghai Institutes for Biological Sciences, CAS, Shanghai 200031, China

<sup>c</sup> Graduate School of the Chinese Academy of Sciences, China

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### Abstract

This is the introduction of *Drosophila* into the study of learning and memory affected by removal of the geomagnetic field (GMF) for successive generations. Using the operant visual learning/memory paradigm at a flight simulator, the present study revealed that wild-type flies raised in a hypomagnetic field environment continuously for 10 successive generations were gradually impaired in visual conditioning learning and memory formation and finally the 10th generation flies became morphs of nonlearners and completely amnesiac. The control experiments show that the impairment could not be ascribed to any apparent sensorimotor problems in *Drosophila*. The reverse shift from hypomagnetic field (HMF) to natural GMF restored the GMF-free induced amnesia fully after six consecutive generations. Thus, our findings demonstrate conclusively that some serious, but reversible learning and memory impairment may occur for living organisms in a prolonged separation from GMF over many consecutive generations. And *Drosophila* has the potential to develop into a new model organism for the study of the neurobiology of magnetism for multiple generations.

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Previous study has established a definite relationship between the most diverse properties of living organisms and earth's geomagnetic field (GMF) [4]. A recent study has shown that an environment with a hypomagnetic field (HMF) caused a significant negative effect on memory formation in day-old chicks [16]. Whether HMFs affect the learning and memory ability for successive generations is unknown. It is difficult to test the effects of HMFs on learning and memory (L/M) for multiple generations with animals that have a long lifespan. *Drosophila* has a short lifespan (the average lifespan is about 50 days after eclosion), and they reproduce about 10 days after birth, whereas humans reproduce about 10,000 days after birth [12]. The molecular architecture

of vertebrate and invertebrate nervous systems is mostly shared [3,18]. *Drosophila* has most – if not all – of the major neurotransmitters and molecules involved in synaptic vesicle release and recycling, receptors and channels necessary for neurotransmission as well as signal transduction mechanisms. Moreover, *Drosophila's* nervous system is many orders of magnitude simpler than that of mammals, and its genetics are comparatively more sophisticated and rapid. Therefore, *Drosophila* has the potential to develop into a novel model organism for the study of the neurobiology of HMFs for multiple generations [1].

The compensated HMF space was produced by three pairs of Helmholtz coils 2.01, 1.80 and 1.61 m in diameter intersecting one another vertically and by compensating the values of the geomagnetic field in vertical, north and south, and east and west direction, respectively. The value of the residual geomagnetic field resulting from this was 100–680 nT (the

\* Corresponding author. Tel.: +86 10 64888532/86 21 54921785; fax: +86 21 54921735.

E-mail address: [akguo@ion.ac.cn](mailto:akguo@ion.ac.cn) (A. Guo).

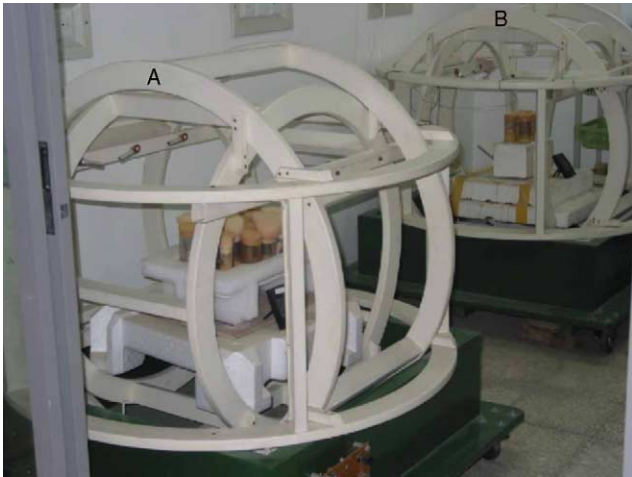


Fig. 1. The HMF system: (A) HMF flies and (B) control flies.

natural GMF in our laboratory is about 52207.02 nT). There were two identical sets of Helmholtz coils in the same air-conditioned room; one was used throughout the whole study for growing experimental flies, while the other one was not switched on for control flies, so that both groups of flies would have been exposed equally to all external influences, such as temperature, humidity and light/dark regimes (Fig. 1).

Visual operant conditioning of flies was tested in the natural GMF environment using a flight simulator, which has been described before [2,19]. Briefly, in this process the test fly is firmly attached by its thorax and head to a hook. It is surrounded by a translucent drum with visual patterns on its periphery. The fly's yaw torque is recorded and is transformed continuously and instantaneously into the negative angular velocity of the drum (negative feedback loop). The visual stimulus consists of two upright and two inverted T-shaped figures (total horizontal and vertical width of figures 40°, of bars 8°) in the four quadrants of a brightly illuminated arena, with identical patterns facing each other. The fly stabilizes the panorama and chooses its flight direction with respect to the visual patterns. For conditioning, certain pattern orientations are associated with heat shock (CO<sub>2</sub> laser beam ( $\lambda = 10,060$  nm) as a punishment stimulus). The heat shock is switched on whenever these particular patterns enter the frontal 90° sector of the fly's visual field. Flies quickly learn to avoid pattern orientations associated with heat shock and retain the respective pattern preferences even after heat shock is switched off.

Performance indices ("preference index" before training, "learning index" during training, and "memory index" after the training) are calculated as  $PI = (t_c - t_h)/(t_c + t_h)$ , where  $t_c$  is the time the fly is oriented toward a sector not associated with heat and  $t_h$  is the time the fly is oriented toward a sector that, during training, is associated with heat. Background luminance  $I = 400$  cd/cm<sup>2</sup> in the experiment. In all instances, avoidance and learning indices were not corrected for the spontaneous pattern preferences of the individual flies during the pre-training session.

The experimental flies were grown under the active coils (Fig. 1A), and the control flies under the inactive coils (Fig. 1B), separately and simultaneously. Unless stated otherwise, 3- to 4-day-old females of wild-type Berlin (WTB) were used. Flies were grown on a standard cornmeal/molasses medium at 25 °C with 60% humidity in one 12:12 light/dark cycle [6]. The experimental flies were transferred to fresh food vials 0–24 h after hatching. Then they were separated and a hook was glued to their head and thorax. Each fly was put into a separate cylindrical bottle (10 mm in diameter and 20 mm deep) where it stayed with a single hook attached to its back. Experiments were carried out on single flies one after another between 08:00 and 20:00 h the following day. Most test flies were 48- to 72-h-old flies. Within these ranges of age and training as well as testing times, no differences were detected in flies' conditioned performance [6].

To evaluate visual memory retention, the WTB flies raised in the HMF environment were conditioned in a flight simulator with the standard conditioning procedure [7]. In the 24-min protocol, the test fly was first examined during a pre-training test period (three 2-min blocks as PI<sub>1–3</sub>) for its directional preference for various patterns in the arena. This was followed by two training sessions (two 2-min blocks each, as PI<sub>4–5</sub> and PI<sub>7–8</sub>, spaced by one 2-min test block of PI<sub>6</sub>) during which the heat shock was switched on whenever a particular pattern entered the frontal 90° sector of the fly's visual field. The post-training test sessions consisted of four 2-min blocks (PI<sub>9–12</sub>) without heat shock application.

Fig. 2A shows the mean PI<sub>4–5</sub> and PI<sub>7–8</sub> during training period and PI<sub>9–12</sub> measured immediately after the second training session (PI<sub>8</sub>) as a function of generations, respectively obtained from the total quantity of 445 flies; for each generation more than 18 flies were tested. Each test fly was used in only one experiment.

The results in Fig. 2 show that the impairment in short-term memory retention PI<sub>9–12</sub> began to be statistically significant in the sixth generation of HMF flies (HMF6) ( $P < 0.01$ ) (Fig. 2C). And it continued to become progressively worse until complete amnesia appeared in the 10th generation of HMF flies (HMF10) ( $P > 0.05$  for the last four PIs versus zero) (Fig. 2D).

The results in Fig. 2A also show that in parallel to the abnormal short-term memory retention performance, we found similar significant impairment in heat shock avoidance indices (learning indices) PI<sub>4–5</sub> and PI<sub>7–8</sub> in the second training session. The learning index (PI<sub>7</sub> and PI<sub>8</sub>) of HMF6 flies during training was lower than that of the control flies (Fig. 2C), and it became progressively worse with subsequent generations (Fig. 2D and E). This observation indicated that the HMF caused defects not only in short-term memory performance, but also in the acquisition (learning) process (Fig. 2A), and these defects could not be improved during the course of the training. This indicated that associative learning did not happen between conditioning stimulus (CS: visual patterns) and unconditioning stimulus (UCS: heat

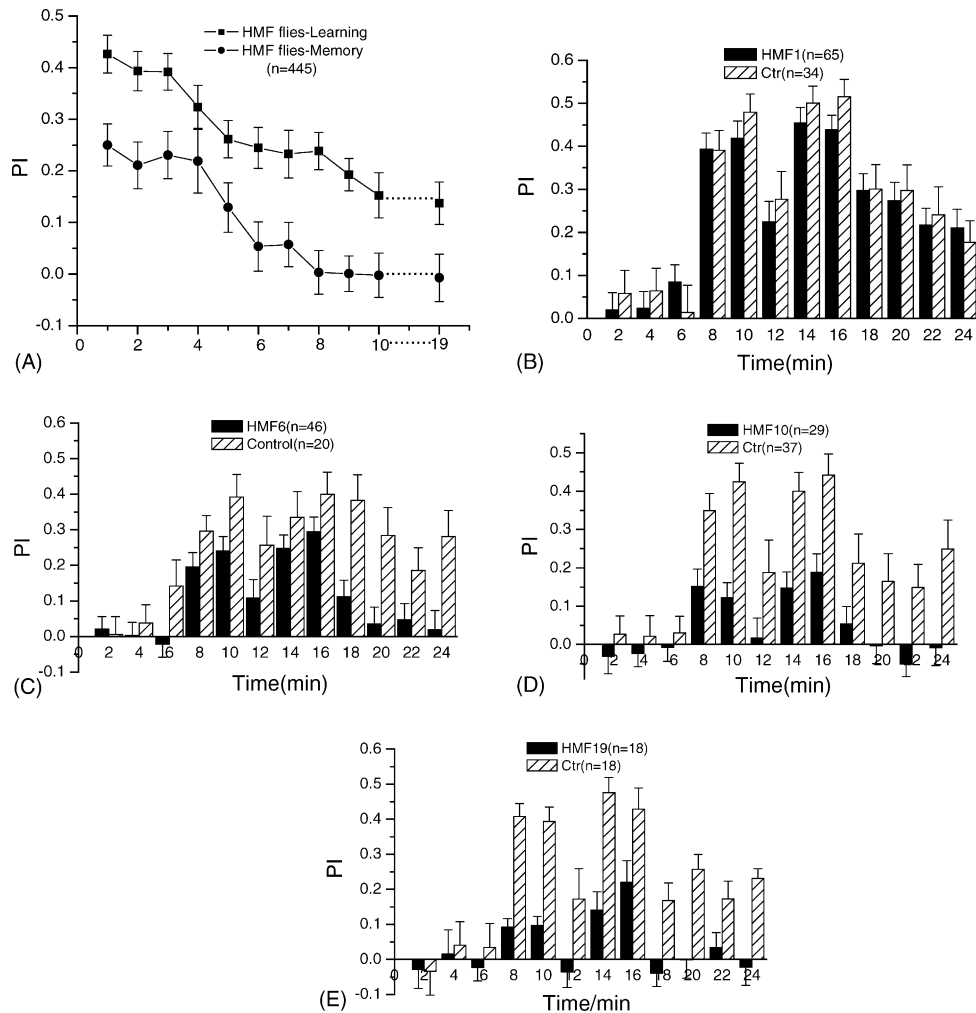


Fig. 2. Visual learning and memory impairment of flies raised in a hypomagnetic field (HMF flies) for 19 successive generations. (A) The reduction curve of the performance index (PI) of operant visual learning and memory (L/M) formation of flies showed both L/M PI decreased with the increase of generation from the first to the 19th generation. Data point (■) represents mean  $PI_{4-5}$  and  $PI_{7-8}$ . Data point (●) represents mean  $PI_{9-12}$ . Error bars in all figures in this paper indicate S.E.M.  $n$  refers to the number of flies (S.E.M.;  $n = 18$  to 65 for each generation). (B) The performance of the first generation of HMF flies (HMF1) in the 24-min standard protocol. L/M PI of HMF1 flies did not differ significantly from that of control flies ( $t = 1.077$ ,  $P > 0.05$ ;  $t = 0.070$ ,  $P > 0.05$ ). (C) The learning process of the sixth generation of HMF flies (HMF6) in the 24-min standard protocol. The statistically significant impairment in short-term memory measured immediately after the second training session ( $PI_8$ ) began to appear in HMF6 compared to that of the flies raised in the natural GMF (control flies). The difference of memory PI between the two fly populations was significant ( $t$  test,  $t = 3.304$ ,  $P < 0.01$ ). (D) The 10th generation of HMF flies (HMF10) subjected to the 24-min training procedure exhibited complete amnesia. No memory was detected in these flies. The memory PI (mean  $PI_{9-12}$ ) did not differ significantly from zero ( $t = 0.071$ ,  $P > 0.05$ ). The differences of L/M PI between HMF10 and control flies were statistically significant ( $t = 5.86$ ,  $P < 0.001$ ;  $t = 3.585$ ,  $P < 0.001$ ). (E) In the 24-min procedure no memory was demonstrated in the 19th generation of HMF flies (HMF19). The difference of L/M PI between HMF19 and control flies was significant ( $t = 7.723$ ,  $P < 0.001$ ;  $t = 5.531$ ,  $P < 0.001$ ). And the memory PI did not differ significantly from zero ( $t = 0.244$ ,  $P > 0.05$ ).

shock) during the training session for HMF flies. It is well known that the efficacy of conditioning depends on establishing CS–UCS links [7,14].

In order to address whether the fully amnesiac HMF10 flies could produce offspring free of amnesia by natural GMF, we transferred HMF10 flies into the natural GMF (transferred flies) (Fig. 3).

We found that the reverse shift from HMF to natural GMF reversed the GMF-free induced amnesia in later generations, and the complete restoration to a stationary state required about six consecutive generations under the natural GMF

(Fig. 3A). Fig. 3B shows that the transferred 6th and 10th generation flies have normal learning and short-time memory ability compared to control group.

Finally we carried out a series of behavioral control experiments to rule out the possibility that the L/M impairment of HMF flies was a secondary effect of sensorimotor problems resulting from the HMF.

It is well known that with the visual operant conditioning paradigm at the flight simulator, flies can only be trained successfully when they can normally perceive and recognize the visual patterns and correctly avoid the heat shock, and asso-

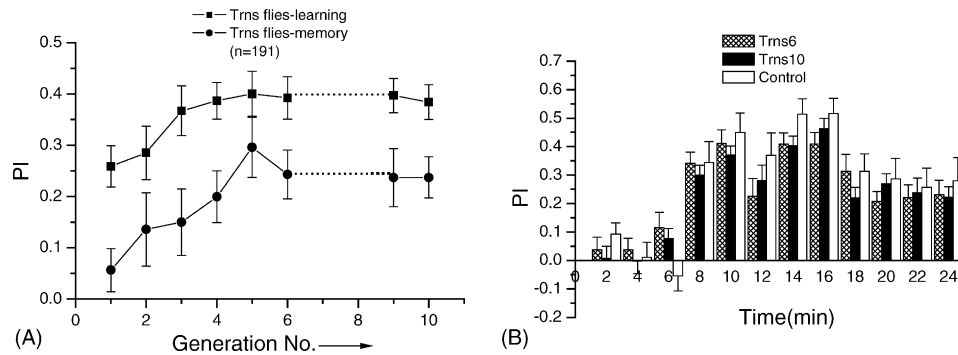


Fig. 3. Visual learning and memory restoration after HMF flies were transferred into natural GMF (transferred flies). (A) The recovery curve of visual L/M formation for transferred flies showed that L/M PI increased gradually from generation to generation (S.E.M.;  $n = 20$  to  $29$  for each generation). In parallel to the recovery memory retention performance, we found a similar significant recovery in learning indices  $PI_{4-5}$  and  $PI_{7-8}$  in the training sessions. (B) The learning process of transferred flies in the 24-min standard protocol. There were no significant differences in L/M PI for the 6th and 10th generations of transferred flies (Trns6 and Trns10) and control flies (ANOVA,  $F = 1.547$ ,  $P > 0.05$ ;  $F = 0.422$ ,  $P > 0.05$ , respectively).

ciate the CS with the UCS [7,14]. In this regard, we carried mainly three control experiments.

To figure out whether the defective L/M demonstrated by HMF flies resulted from the defect in pattern discrimination performance, the spontaneous visual pattern preferences were examined (Fig. 4A). As mentioned above, during the pre-training session of the standard conditioning protocol, three PIs ( $PI_{1-3}$ ) were calculated for each test fly, which could be used to characterize the fly's spontaneous preference between the two different visual patterns [21].

Of the  $PI_{1-3}$ , we selected the one with the maximal absolute value and defined this value as the spontaneous pattern preference index (PPI) of each individual fly. Thus, the PPI indirectly hints at the visual pattern discrimination ability of the test fly. All 111 control flies and 185 HMF flies were tested in the pre-training session. The PPIs showed that both HMF and transferred flies sense and distinguish the two different visual patterns normally and change their pattern preference randomly.

These results indicated that the L/M defect cannot be attributed to the possible damage in the spontaneous pattern discrimination ability in HMF flies.

It is well known that visually induced behavior occurs whenever a contrasted object moves relative to the fly's visual field. This is called object tracking in front of a background [9]. It has been shown that the learning mutants *dnc* and *amn* have apparently normal vision, but they could have a visual attention deficit in term of object tracking [5,20]. To address whether the L/M defect under HMF space for successive generations could have resulted from a visual attention defect of HMF flies, we used the single-target tracking paradigm [22]. The fly's flight direction relative to a vertical black stripe was recorded continuously at a sampling frequency of 20 Hz. At the same time, the fly's direction (from  $-180^\circ$  to  $180^\circ$ ) relative to the object was converted into the error angle  $\psi$ . The error angle ( $\psi$ ) is defined as ( $\alpha_p - \alpha_f$ ) and represents the angular position of the fly with respect to the object in the fly's coordination system (inset in Fig. 4B).  $\alpha_f$  represents the

instantaneous direction of flight with respect to an arbitrary zero-direction, and  $\alpha_p$  represents the instantaneous angular position of an object that the fly may or may not track. When  $\alpha_p = \alpha_f$ , the fly's long axis points directly at the tracked object ( $\psi = 0$ ). The characteristic variable in these experiments was the 'error angle'  $\psi$ . The average of the absolute value of the error angle ( $|\psi|$ ) during each experiment was used as an error index to describe the orientation performance (Fig. 4B). The HMF flies showed visual attention ability as good as the control flies. These results exclude the involvement of the possible visual attention defect in the L/M of HMF flies, allowing the assumption that the flight control of these HMF flies was not defective.

Upon perception of a noxious stimulus, an organism executes defensive mechanisms, such as avoidance response [13,17]. Heat shock has been used as an effective negative reinforcing stimulus in operant visual conditioning in the past decade. To test whether HMF flies are thermoblind to noxious heat shock, we carried out a control experiment to assay the temperature sensitivity of HMF flies. Single adult flies were exposed to noxious heat using either a heated iron plate or a monochromatic laser beam ( $\lambda = 10,060$  nm and spot diameter = 1.5 mm) with a power intensity of more than 0.01 W (Cheng Yong Photoelectric Technology, Shanghai Co.). The amount of time before the thermal avoidance response was recorded. These data demonstrate that the HMF flies are not thermoblind and they could avoid the "hot" flight direction and choose the "cold" one correctly (Fig. 4C).

This indicates that L/M defects in HMF flies are not the secondary effects of some sensorimotor problems resulting from HMF.

Here we showed that both acquisition and short-term memory are gradually impaired over multiple generations when GMF is removed from the fly's growing environment. Learning and memory ability also were observed to gradually return to normal levels after successive generations when GMF is restored again to the fly's environment. Cumulative

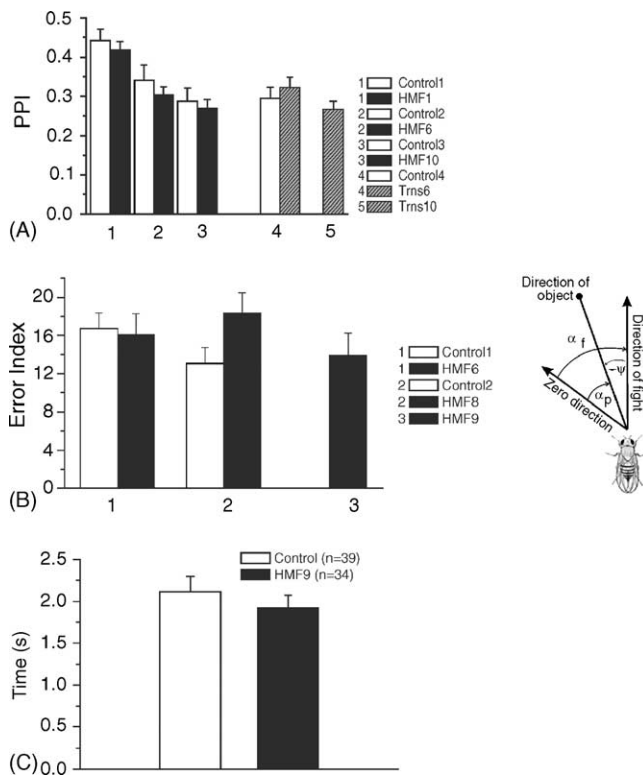


Fig. 4. The following three control experiments show that the impairment of L/M cannot be ascribed to any apparent sensorimotor problems in HMF flies such as the spontaneous visual pattern discrimination behavior, attention-like target tracking behavior, as well as thermal sensitivity to noxious heat shock. (A) Comparisons of pattern preference index (PPI) of different generations of HMF flies or transferred flies with control flies showed that no statistically significant differences existed for five group experiments (as shown in legend) ( $t = 0.679$ ,  $P = 0.499$ ;  $t = 0.911$ ,  $P = 0.366$ ;  $t = 0.566$ ,  $P = 0.574$ ;  $t = 0.673$ ,  $P = 0.505$ ;  $t = 1.604$ ,  $P = 0.116$  (for Trns6 and Trns10) respectively). (B) HMF did not affect the fly's attention-like target tracking ( $F = 1.050$ ,  $P > 0.05$ ).  $n > 14$  for each group. The angle  $\psi = \alpha_p - \alpha_f$  is the "error angle" between the fly's direction of flight and the object. The average of the absolute value of the error angle ( $|\psi|$ ) during each experiment was used as an error index to describe the orientation performance. (C) The performance of flies with the hot laser beam paradigm. The time delays of the temporary escape response initiated by infrared laser beam between control and HMF9 flies were not significant different ( $t = 0.823$ ,  $P > 0.05$ ).

environmental effects over several generations in *Drosophila* have been reported before. Ho et al. [8] elicited phenocopies of *bithorax* after treatment with ether and observed that the penetrance and expression of these phenocopies increased in successive generations. They ruled out selection and offered "cumulative cytoplasmic modification" as the most likely explanation. Guo et al. [6] observed a gradual loss (and gain) of learning ability in *Drosophila* over several generations after a dramatic change in food quality.

How are the gradual changes in learning and memory behavior over several generations manipulated by removing GMF? What is the underlying mechanism by which the magnetic-field-free induced impairment in learning and memory could be transferred and accumulated from generation to generation, and finally result in complete amnesia?

And what is the reverse mechanism by which the learning and memory could be restored?

In many species environmental factors can trigger different developmental programs or deviation from the same developmental program. The best example is that rover and sitter fruitfly larvae show different patterns of foraging-related behavior when searching for food [10,11]. Both rovers and sitters should be considered as wild-type phenotypes as they are maintained in nature at appreciable frequencies, and the natural selection can shift the ratio of rover and sitter individuals in the population in a density-dependent manner [11].

In the present case, the loss and gain of learning over generations as the function of the parameter of GMF suggests that a genetic selection might underlie this effect. We would like to assume that there are two developmental programs leading to the two "morphs" (i.e., learners or nonlearners), and these programs would be transmitted to the next generation. Both learners and nonlearners should be considered as wild-type phenotypes as they are maintained in nature at appreciable frequencies in the population. The bimodality of the learning ability in flies indicates that both learner and nonlearner types might be maintained by natural selection, and GMF as the trigger factor can shift for allelic frequencies such that learners are selected in the presence of GMF environments and nonlearners in the absence of HMF ones.

These normal individual differences in learning behavior could be explained by variation in a single gene called *dunce* (*dnc*), the first single-gene mutant for associative learning. The *dnc* gene is a large, complex locus that has several transcripts and pleiotropic effects during development and adulthood [3,11]. The adult flies with learner alleles can be made to behave as nonlearners after multiple generations in the absence of GMF. Conversely, those with nonlearner alleles can be made to behave like learner alleles in the presence of GMF. So, both intrinsic and extrinsic factors influence the behavior expression in learners and nonlearners. So far we do not know how to describe it in molecular terms, but it is certainly related to the cAMP signaling pathway [15].

The present study demonstrates, for the first time, that WTB flies raised in an HMF environment continuously for 10 successive generations were gradually significantly impaired in visual L/M and finally the 10th generation of HMF flies became completely amnesiac. The reverse shift from hypo- to natural-GMF fully reversed the GMF-free induced amnesia after more than six consecutive generations. The control experiments show that the impairment of L/M could not be ascribed to apparent sensorimotor problems in HMF flies. This study suggests that *Drosophila* have the potential to develop into a new model organism for the study of neurobiology of magnetism for multiple generations. We are inclined to believe that our study may be a novel example of experimental evolution in the laboratory, in which flies with learner or nonlearner alleles were selected depending on the presence or absence of the geomagnetic field.

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