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Zebrafish respond to the geomagnetic field by bimodal and group-dependent orientation

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A variety of animals use Earth's magnetic field as a reference for their orientation behaviour. Although distinctive magnetoreception mechanisms have been postulated for many migrating or homing animals, the molecular mechanisms are still undefined. In this study, we found that zebrafish, a model organism suitable for genetic manipulation, responded to a magnetic field as weak as the geomagnetic field. Without any training, zebrafish were individually released into a circular arena that was placed in an artificial geomagnetic field, and their preferred magnetic directions were recorded. Individuals from five out of the seven zebrafish groups studied, groups mostly comprised of the offspring of predetermined pairs, showed bidirectional orientation with group-specific preferences regardless of close kinships. The preferred directions did not seem to depend on gender, age or surrounding environmental factors, implying that directional preference was genetically defined. The present findings may facilitate future study on the molecular mechanisms underlying magnetoreception.

Many animals are capable of sensing Earth's magnetic field and can most likely use this information for orientation in migration, homing, escaping and nest building. Magnetosensitivity has been demonstrated in mollusks, insects, crustaceans and all major groups of vertebrates[1]. The biophysical mechanisms underlying magnetoreception are poorly understood, although three mechanisms have been proposed to date[2]: stimulation by electromagnetic induction[3]; a process based on magnetic minerals such as magnetite[4,5]; and a radical pair reaction involving photoreceptors[6,7] such as cryptochromes (CRYs), UVA- and blue light-absorbing photoreceptors that contain the flavin adenine dinucleotide (FAD) chromophore. Recent studies have suggested that both the magnetic mineral and radical pair reaction mechanisms coexist in vertebrates, with varying predominance and function depending on the animal species and its lifestyle[8,9].

Magnetoreception research began with behavioural studies of long-distance migratory or homing animals such as salmon, newts, sea turtles, passerine birds and homing pigeons, and of late some of these animals have also been used for lab study[9,10]. These animals, however, do not function as the best systems for molecular analysis of magnetoreception because of the lack of convenience of genetic manipulation compared to model animals. Based on the recent use of mutants or genetically engineered fruit flies, the fruit fly *Drosophila melanogaster* has been found to be an excellent model for molecular analysis of magnetoreception[11]. Mutant flies lacking normal CRY did not exhibit the same behavioural response to a magnetic stimulus that is seen in wild-type flies, strongly suggesting that CRY may function as a light-sensitive magnetosensor. Whether vertebrate CRYs have the same function remains unclear, but recent findings suggested that the transgenesis of human CRY2 into mutant flies compensates magnetoreception ability[12].

Various characteristics of zebrafish (*Danio rerio*) make them one of the best vertebrate models for the study of development and toxicology, and likewise they may also be suitable for the molecular analysis of vertebrate magnetoreception. While the magnetosensitivity of zebrafish has been suggested[13], previously conducted magnetic field trials varied magnetic field intensity using a pair of coils placed close to the test compartment. Therefore, the possibility that the magnetosensation was mediated by electric signals induced by the change in magnetic field near the coils could not be ruled out. Also, it remains unclear whether zebrafish can actually respond to a magnetic field as weak as the Earth's because an artificial magnetic field stronger than the geomagnetic field was used. In order to address these issues, we developed another experimental design in which the
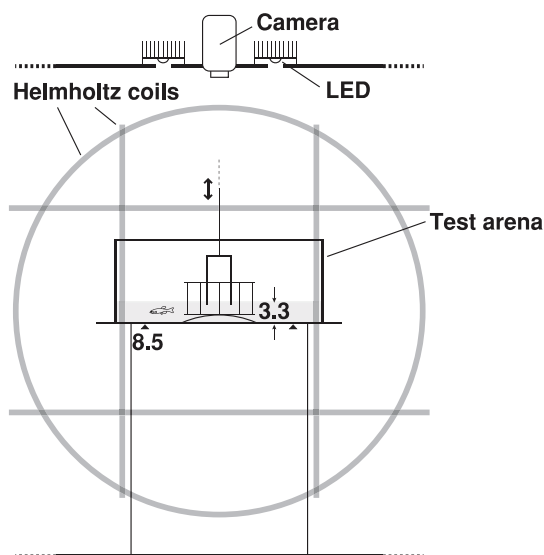
Figure 1 | The experimental setup. A manually controlled cylindrical release device was centered in a water-filled tub (Test arena) that was placed on a foam polystyrene block and enclosed within three-axis Helmholtz coils in a wooden box. A web camera was centered over the release device. LEDs were placed concentrically around the camera equidistant from each other.

magnetoreception of zebrafish was examined using a static magnetic field. Zebrafish showed bidirectional orientation to particular magnetic directions in artificially modified magnetic fields as weak as the geomagnetic field, and it could be possible that the magnetic preferences were defined by the genetic backgrounds of the fish. We also provide along with the findings of our study a discussion of the physiological importance of the bidirectional- and group-specific response seen in the zebrafish.

Results

Under the artificial magnetic field as strong as geomagnetic field, each zebrafish was individually placed into the test arena (Fig. 1) to record its motion, and the video data obtained from one group of fish were analyzed after the experiments. Initially, we used fish that were the mixed offspring of more than five couples from the zebrafish Ekkwill strain (Group I, Fig. 2). They did not show any preference towards a specific direction during the unimodal analysis (p = 0.766; Table 1, Fig. S1) but did produce significant results under a bimodal distribution with preference towards magnetic northeast or southwest (52°/232°, r = 0.445, n = 21, p = 0.014; Group I, Table 1, Fig. 3).

Group II, comprised of a unified population with the same mother but different father (Fig. 2), showed bimodal orientation with an averaged direction of orientation (142°/322°, r = 0.486, n = 13, p = 0.043; Group II, Table 1, Fig. 3) that diverged markedly from that observed in Group I (52°/232°; Group I, Table 1, Fig. 3). We considered several possibilities for this divergence (see discussion), and commenced testing zebrafish groups with closely related yet slightly different genetic backgrounds in the experiments to follow; Five groups of fish (Group III to VII; Fig. 2), each containing offspring from a predetermined couple from the other fish groups. In bimodal analyses, all groups except for Group IV showed group-specific orientation (Table 1, Fig. 3). Group III oriented itself magnetic east or west (82°/262°, r = 0.338, n = 27, p = 0.044; Table 1, Fig. 3), and Group VI oriented itself magnetic north or south (165°/345°, r = 0.395, n = 21, p = 0.036; Table 1, Fig. 3). Only Group IV, consisting of cousins from Group VI, showed random distribution (n = 13, p > 0.60; Table 1, Fig. 3). Group V, an offspring group of a predetermined pair from Group IV, exhibited a significant distribution of magnetic bearings due roughly north or south (160°/340°, r = 0.356, n = 25, p = 0.040; Table 1, Fig. 3). Group VII, an offspring group of a predetermined pair from cousins from Group IV and Group VI, exhibited a significant distribution of magnetic bearings due roughly north or south (172°/352°, r = 0.323, n = 36, p = 0.022; Table 1, Fig. 3) and resembled the magnetic bearings of Group V and Group VI (all pairs among Group V, Group VI and Group VII; p > 0.50, Watson's U^2-test). In order to evaluate these results considering the problems of multiple testing, we performed statistical analysis using Benjamini and Hochberg method (BH method)[14], which gave p-value nearly equivalent to 0.05. In unimodal analyses, all groups except Group VI exhibited random distribution (Table 1, Fig. S1); only Group VI oriented itself magnetic southward (158°, r = 0.458, n = 21, p = 0.011; Table 1, Fig. S1), but p-value corrected for multiple testing by BH method was not less than 0.05.

We additionally analyzed latency time (Fig. S2) and special behaviour (Fig. S3) until end of the trial for each individual. More than 70 percent of fish ended the trial within 5 sec and a median of the latency times was 2 sec (Fig. S2). Although the spatial behaviour did not always correlate with the direction of magnetic bearings, many fish showing relatively short latency times swam to cross over the 8.5 cm-radius-circular line just after the release (Fig. S3).

Discussion

The present results indicated that zebrafish (Ekkwill strain) have a preference for particular magnetic directions, and the distributions of their magnetic bearings diverged by population even within a
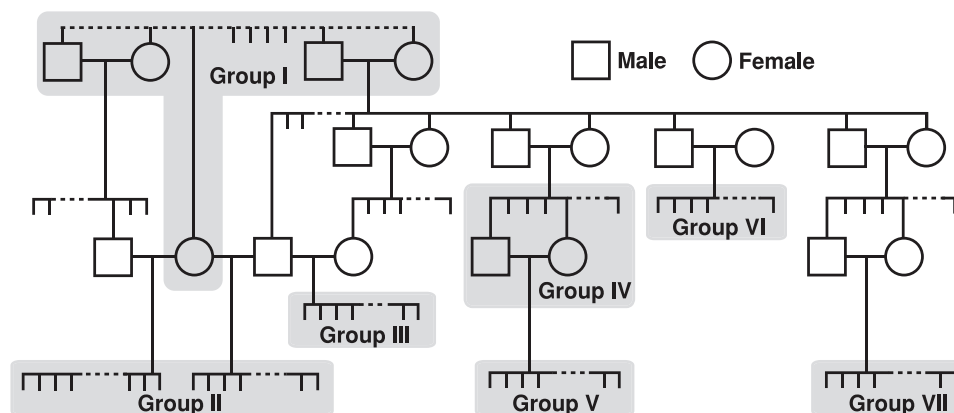

Figure 2 | Family tree of tested zebrafish. Group I consisted of the mixed offspring of more than five couples from Ekkwill strains. Group II was a unified population from the same mother but different father. Group III to VII were offspring from the zebrafish in Group I.

Table 1 | Age and number of tested zebrafish[+]

Fish group	Age (Month)	Number (Male:Female)
I	23	21
II	5	13
III	11	27 (18:9)
IV	5	13
V	9	25 (14:11)
VI	14	21 (21:0)
VII	17	36

[+]Age, ages when tested. Groups I, II, IV and VII were tested without regard to gender.

Figure 3 | **Magnetic orientation of zebrafish Groups I-VII.** Bimodal distributions of each group. Each black arrowhead represents the bearing of one zebrafish. A gray arrowhead was drawn at the opposite side of each black arrowhead. A double-headed arrow at the center of each plot indicates the mean vector of each distribution. The length of each arrow is proportional to the mean vector length (r), with the diameter of the circle corresponding to r = 1. Large black triangles outside the plots indicate magnetic north.

single family (Fig. 2, Table 1). Several possibilities may be causing the different preferences in orientation.

Our initial assumption was that the preference of direction depended on differences in time or season or age. In the case of newts, for example, magnetic orientation changes seem to depend on age or season[15]. However, based on the setup of our study, this assumption seems unlikely since we performed all of the present experiments at the same time of day (13:30–16:30), and all zebrafish groups were maintained under a fixed light/dark cycle in water that was the same temperature. Concerning age, the Group II and Group IV zebrafish tested at 5-months of age showed different distributions from each other (Fig. 3, Table 1). Therefore the group-specific preference in orientation is probably independent of age.

Next, we considered differences in gender, since a gender effect has been reported in magnetic compass orientation of *Drosophila*[16] and mice[17]. Gender-related differences are also unlikely since there is significant difference in the orientation between males and females in neither Group III nor Group V (p > 0.50, Watson's U^2-test, Fig. 4, Table 2). Also we did not find any gender effect when all the males and females in Group III, V, and VI were combined (p > 0.50, Watson's U^2-test).

Lastly, we investigated the possibility that zebrafish learned to swim towards a particular magnetic direction based on environmental cues such as the magnetic direction of tank placement and/or surrounding light conditions. Eastern red-spotted newts, *Notophthalmus viridescens*, were shown to learn magnetic directions and to orient themselves toward the direction of the shore or deep water[18]. Therefore, we thought it plausible that zebrafish might avoid the bright (or dark) experimental environment and learn to swim towards the magnetic direction of the dark (or bright) side of the tank or that they might also use magnetic cues to assess their geographic location. Under our experimental conditions, the tanks were placed in a similar magnetic environment with only slight differences (Table S1). During the light period, all breeding tanks were illuminated under a fluorescent lamp so that the magnetically southern side was brighter than the northern side. Magnetic preferences of the zebrafish diverged to include many directions, even the magnetic east-west direction (Group III, Fig. 3), and there was no correlation between magnetic direction of the tank and group distribution (Fig. 3, Table S1): Groups II and IV, which had an almost identical field intensity and inclination of field lines (Table S1), ended up having quite different distributions (Fig. 3, Table 1). In addition, the population-dependent social behavior acquired in the bleeding tank is unlikely to drive the group dependency in the present experiments, because we keep the fish of each group separately in multiple tanks. The magnetic bearings of the fish should depend on the bleeding tank rather than the genetic background if it is driven by the social behaviour acquired in the tank. These results lead us to believe that preferred direction might be coded genetically, although we could not rule out other unknown factors.
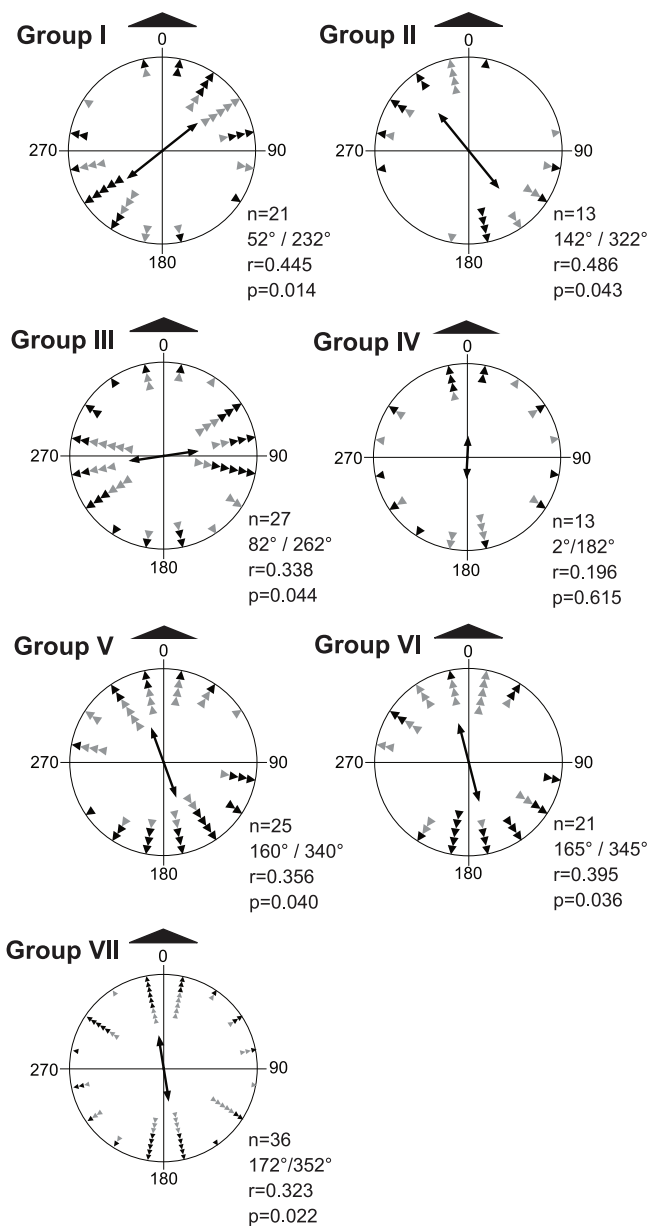
Genetic programming of magnetic navigation systems has been reported in hatchling loggerhead sea turtles, *Caretta caretta*, that need to be able to distinguish magnetic field direction in order to keep within their migratory route, even in territory previously unencountered[19]. Future studies should be undertaken to examine whether magnetic preference is also genetically encoded in zebrafish and to identify the gene(s) contributing to magnetoreception.

Wild zebrafish mainly inhabit the Ganges basin and can also be found in slow moving streams over the course of the year[20]. During rainy season, these areas are repeatedly exposed to heavy monsoon rains, and floodplains appear near the river delta. At the onset of rainy season, adult fish move into flooded areas such as rice paddies
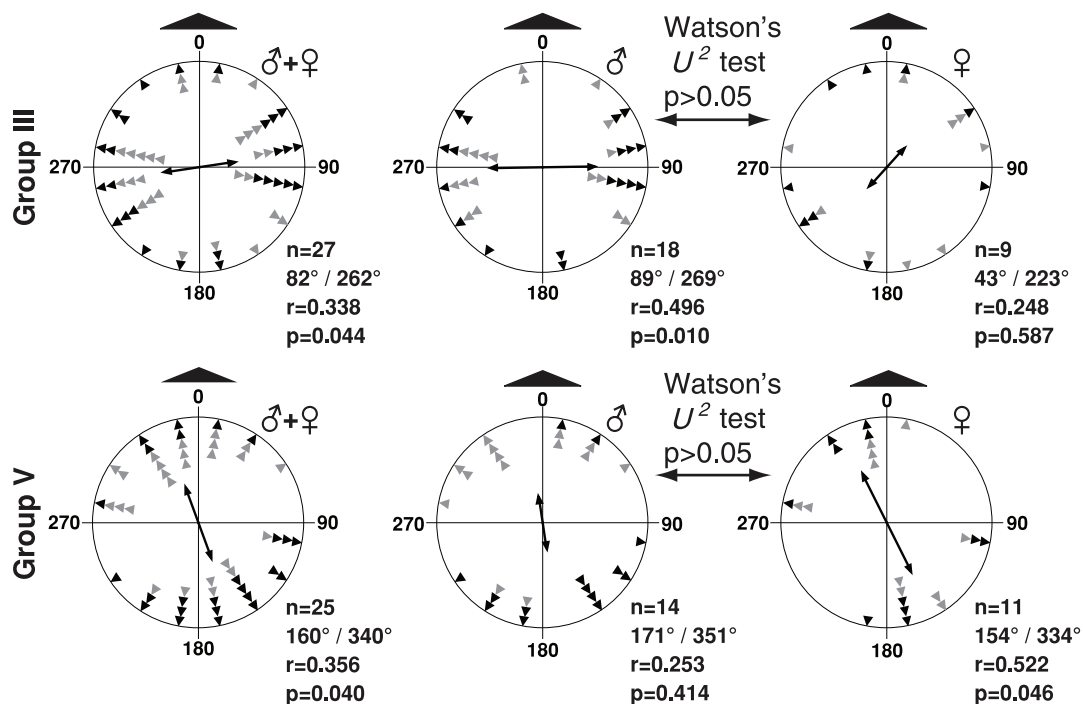
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Figure 4 | Gender differences in magnetic orientation of Group III and Group V zebrafish with bimodal analysis. Bimodal distributions of Group III and Group V are shown in relation to gender differences: (left panel) combined orientation of male and female zebrafish, (center panel) orientation of male zebrafish, and (right panel) orientation of female zebrafish. Each black arrowhead represents the bearing of one zebrafish. A gray arrowhead was drawn at the opposite side of each black arrowhead. A double-headed arrow at the center of each plot indicates the mean vector of each distribution. The length of each arrow is proportional to the mean vector length (r), with the diameter of the circle corresponding to r = 1. Large black triangles outside the plots indicate magnetic north.

for spawning. There are less numbers of predators and abundant foods (plankton) in these seasonal waters, which serve as suitable environments for the development of larvae and juveniles. At the end of the rainy season, young zebrafish move back into the streams as the seasonal waters recede. Thus, zebrafish may migrate back and forth between streams and floodplains. During this migration, since it would be difficult to use olfactory cues from events like the upstream-homing migration of salmon, they may depend on the local magnetic field. Sockeye salmon (smolts), *Oncorhynchus nerka*, are known to use celestial and magnetic cues when moving downstream of the lake to the ocean[21]. The bimodal orientation might help zebrafish to swim for two opposite directions keeping a straight way and increase the efficiency of migrating back to the streams, because the streams and floodplains form irregular and complex patterns. More simply, wild zebrafish may need to use the geomagnetic field for orientation during swimming since the overcast skies during rainy season may reduce the amount of polarized sunlight that passes underwater[22] or heavy rainfall may increase water turbidity and reduce the visibility of landmarks.

Taking into account the environmental and ecological context of wild zebrafish, we can speculate over two possibilities: One is that the randomness of the preferred magnetic direction observed in this study may increase viability during the migration of wild zebrafish

from seasonal waters back to the river streams, because the streams and receding waters are randomly distributed in flooded areas. Another possibility is that the random magnetic direction may help zebrafish to survive against predators. If all fish in a group escape in the same direction, a predator can easily follow the group and have plenty of opportunities for capture. On the contrary, if individuals scatter separately, though it is unlikely in zebrafish[23], each fish may lose recognition and pursuit by initial predators but may increase the chance of being preyed upon by another predator while swimming alone. Thus, the most efficient way to escape predators may be to swim away in two (or a few) different directions, clustering in a group large enough to be able to resist predators. The randomness of the two magnetic directions among independent groups (Fig. 3) would increase their resistance to predators with a geomagnetic sense.

In fish, the primary magnetoreceptor and magnetoreception systems require better characterization. Magnetite crystals have been found in fish bodies[24,25], and multiple genes encoding cryptochromes have been identified in zebrafish in particular[26], implying zebrafish may have two different mechanisms for magnetoreception. To remove the possibility of electromagnetic induction in our study, we created an artificial but static magnetic field similar in strength to the geomagnetic field so that we could isolate geomagnetic bearings in zebrafish. In future study, zebrafish should be screened for

Table 2 | Gender difference and magnetic orientation of Group III and Group V zebrafish with bimodal analysis[+]

Fish group	Male				Female			
	n	μ	r	p	n	μ	r	p
III	18	89° / 269°	0.496	0.010	9	43° / 223°	0.248	0.587
V	14	171° / 351°	0.253	0.414	11	154° / 334°	0.522	0.046

[+]n: number of zebrafish; μ, mean vector bearing of pooled distribution of magnetic bearings; r, mean vector length; p, probability in Rayleigh test. Orientation of each fish is shown in Fig. 4.

mutants lacking magnetosensitivity, and with use of our more efficient and precise screening system, identification of not only the magnetoreceptor, but also its downstream signaling molecules may be possible.

Methods

Animals. All studies were approved by the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University (permission # 08A04, 09A03, 10A03, 2011A074). Male and female zebrafish (Ekkwill strain; bred in-house, Table 1) were used as subjects. The fish were kept in transparent plastic tanks (6.5 × 25 cm, 14 cm water depth) aligned in the same direction (approximately 55° clockwise from geographic north on the long sides of the tanks) and placed on a 14 hr:10 hr light:dark cycle (9:00 lights on) using white fluorescent light (model FHF32EX-N-H, Toshiba). The temperature of the circulating water was kept at 28.0–28.3°C.

Behavioural experiments. All behavioural experiments were performed between 13:30 and 16:30. A tank containing a population of zebrafish was carried into the testing room. For each trial, one zebrafish was individually removed from the tank with a dip net and placed into the test arena (Fig. 1). The arena was symmetrical and included a round plastic tub (diameter = 25 cm, height = 11 cm) with its floor radially marked every 22.5° to allow for scoring of the directional bearing of each fish. The tub was filled with water to a depth of 2 cm above the floor top, and the temperature was maintained at 23.5–26.0°C. After each trial, any visible materials were removed if there are materials floating or sinking in the arena. The arena was illuminated by four white LEDs (model LXK2-PWC4-0180, Lumileds) that were symmetrically placed about 30 cm above the arena floor (503–546 μW/cm², 1.6 k lux). Each fish was put into a white cylindrical release device (diameter = 4 cm, height = 7.5 cm) carefully placed in the center of the arena floor without giving any cue for the magnetic field direction and wait 60 sec for removal of the effect of the setting in the device, the release device was slowly raised after 60 sec, allowing the fish to swim freely within the arena. Each fish's motion was recorded by a web camera (model BSW13K05HWH, Buffalo) located above the release device, and directional response was determined in a blinded fashion by the direction of the fish after first crossing over the 8.5 cm-radius-circular line. Trials were abandoned if fish did not cross over the line within 2 min. Fish are unable to see experimenters during trials. The magnetic field (intensity, direction, and inclination) of the test arena was measured using a 3-axis gaussmeter (model FM-3600, MTI). The intensity and inclination of the environmental geomagnetic field in the laboratory was 35.2 ∼ 35.8 μT and −51.2° ∼ −52.5°, respectively. The horizontal direction of the magnetic field was altered by three-axis Helmholtz coils (diameter = 50, 48, 46 cm) placed around the tub without changing intensity and inclination of the magnetic field. The Helmholtz coils were powered by three power supplies (model ZX-400LA, Takasago). Each fish was examined only once. The naïve fish is tested in one of four symmetrical alignments of the artificially modified Earth-strength magnetic field [i.e., magnetic north (mN) was set to geographic north (gN), gE, gS, gW in cycle]. By putting together the magnetic directional responses of an almost equal number of fish tested in the four different magnetic fields (mN = gN, gE, gS, gW), any possibility of a nonmagnetic directional response was removed from the results of distribution[27]. The preferred direction of each fish was normalized with respect to the direction of magnetic north and all circular statistics were calculated with Oriana 3.0 (Kovach Computing Services). Mean vectors were calculated by vector addition and tested for significance using the Rayleigh test. Statistics for bimodal distributions were calculated by doubling each data value and reducing any value greater than 360 using modulo arithmetic. The Watson's U^2-test was used to test for significant differences between two distributions.

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Author contributions

AT and TF treated the fish, performed the behavioural studies, analyzed the data. TW analyzed the data. AT, MK and TO constructed the experimental apparatus and designed the experiment. AT and TO wrote the manuscript. YK, KO and TO analyzed the data and edited the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

How to cite this article: Takebe, A. *et al.* Zebrafish respond to the geomagnetic field by bimodal and group-dependent orientation. *Sci. Rep.* **2**, 727; DOI:10.1038/srep00727 (2012).