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Supplemental Information

Zebrafish Exploit Visual Cues and Geometric Relationships to Form a Spatial Memory

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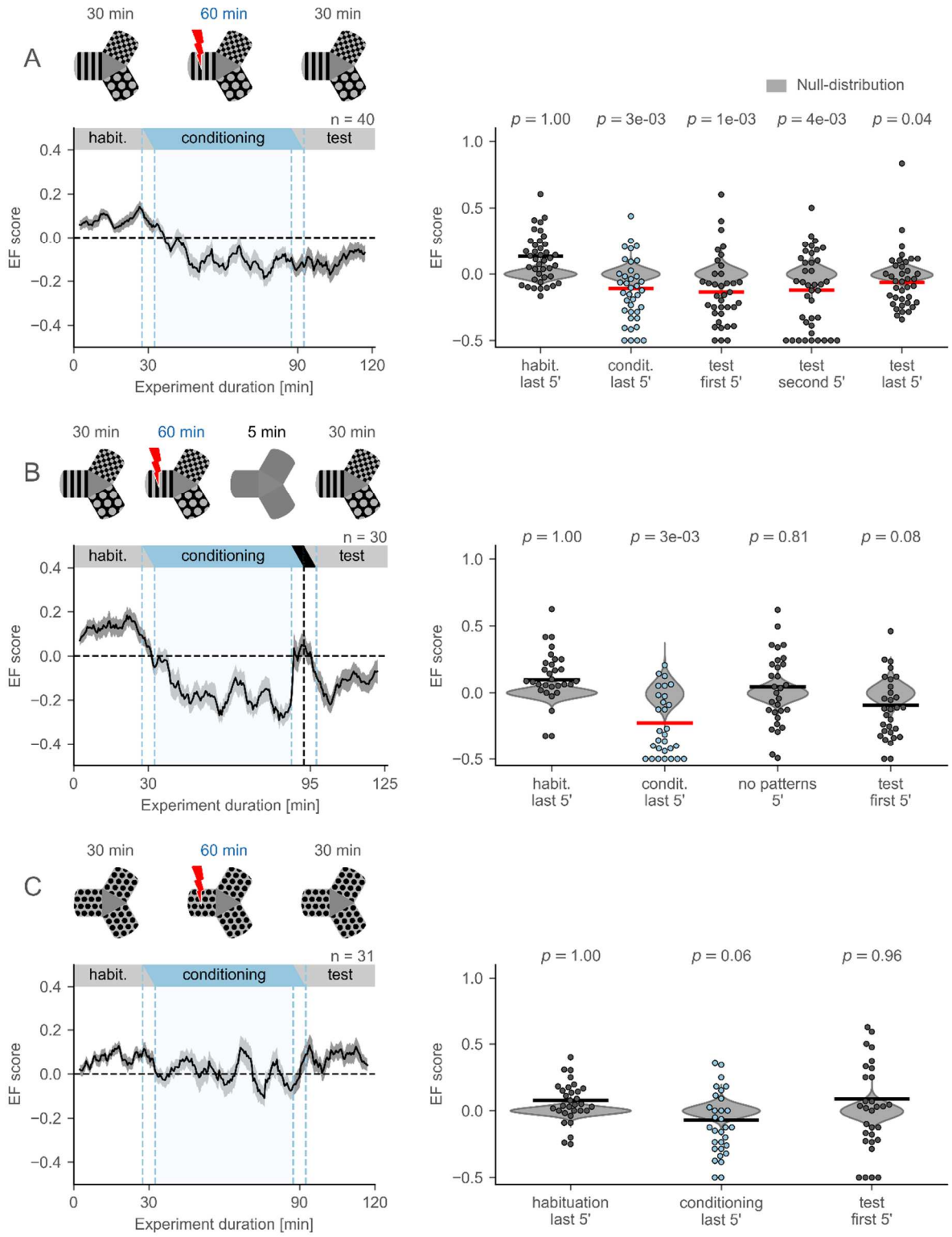

Figure S1. Analysis of the entry frequency score. Related to Figure 3.

(A) Top: schematic of the protocol with habituation, conditioning, and test sessions. Bottom, left: EF score moving average. Right: comparison of EF scores in the last 5 min of conditioning and the first, second, and last 5 min of test session with the null-distribution (permutation test, n = 40 fish). **(B)** Top: schematic

of the protocol with habituation, conditioning, no-pattern, and test sessions. Bottom, left: EF score moving average. Bottom, right: comparison of EF scores in the last 5 min of conditioning, 5 min of no-pattern, and in the first 5 min of test session with the null-distribution (permutation test, n = 30 fish). **(C)** Top: schematic of the protocol where visual patterns in all arms are identical. Bottom, left: EF score moving average. Bottom, right: comparison of EF scores in the last 5 min of conditioning and the first 5 min of test session with the null-distribution (permutation test, n = 31 fish). All annotations are the same as in Figure 3.

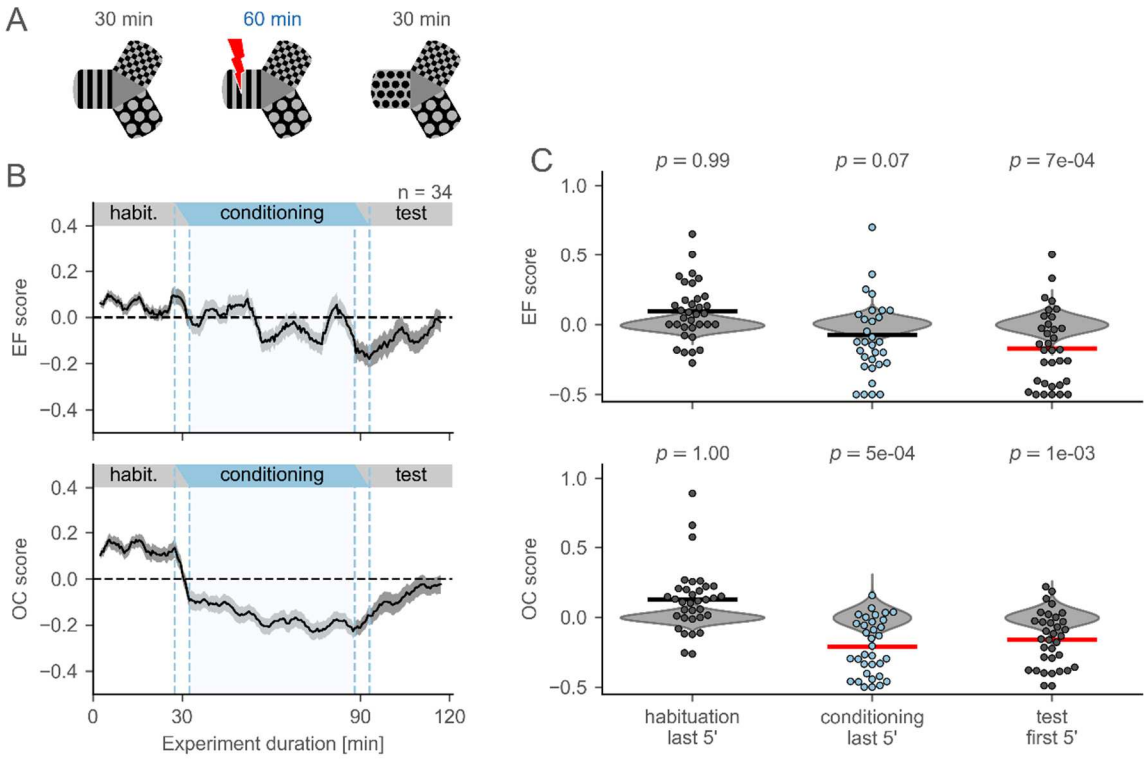

Figure S2. Zebrafish continue to avoid conditioned arm after replacement of conditioned pattern. Related to Figure 5.

(A) Schematic of the protocol with the replacement of the conditioned pattern in the test session. **(B)** EF (top) and OC (bottom) score moving averages. **(C)** Comparison of the EF (top) and OC (bottom) scores in the last 5 min of conditioning and in the first 5 min of test session with the null-distribution (permutation test, n = 34 fish).

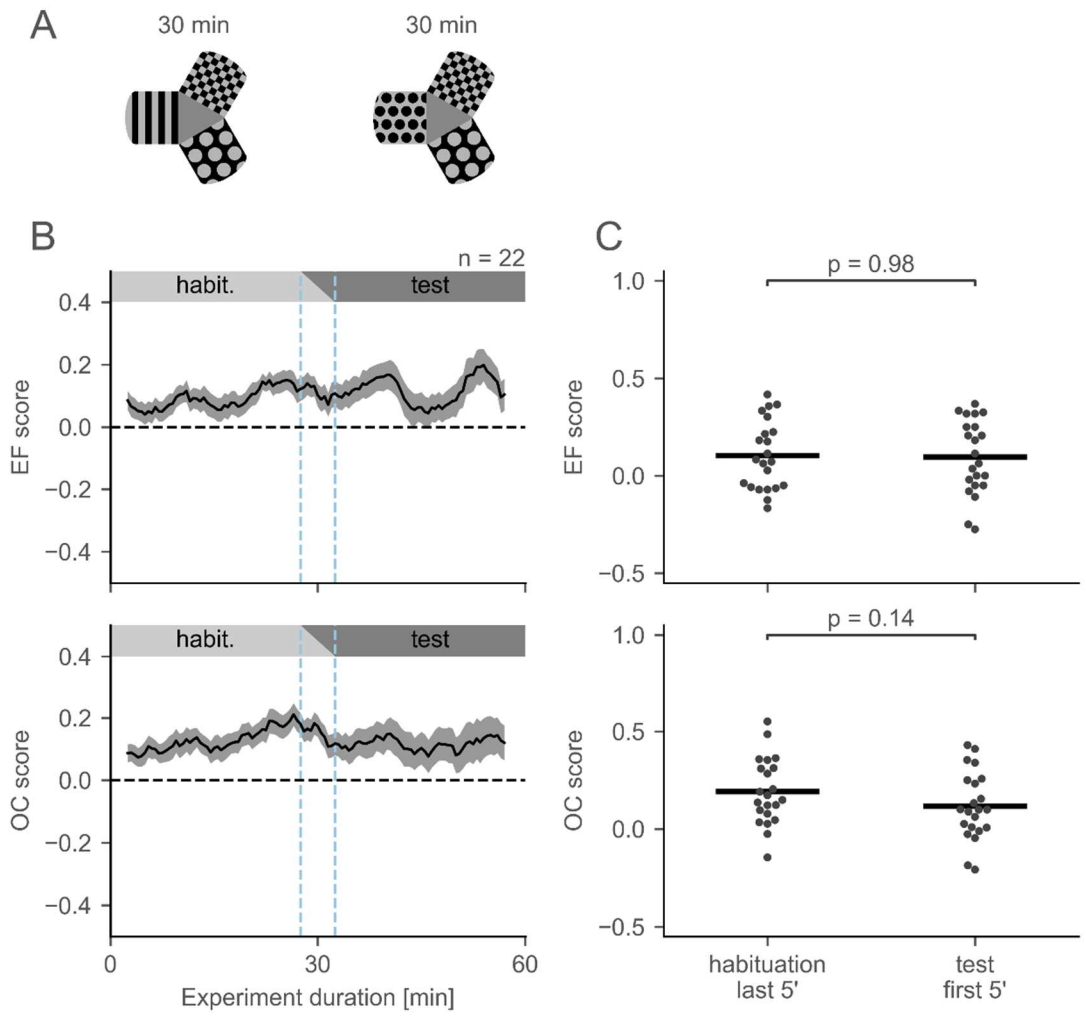

Figure S3. Pattern replacement is neither intrinsically aversive nor attractive to fish. Related to Figure 5.

(A) Schematic of the control protocol with habituation and test sessions. The pattern in the preferred arm was replaced in the test session. **(B)** EF (top) and OC (bottom) score moving averages. **(C)** Comparison of the EF (top) and OC (bottom) scores between the last 5 min of conditioning and the first 5 min of the test session (two-sided Mann-Whitney test, n = 22 fish). Dots show OC/EF score values of individual fish.

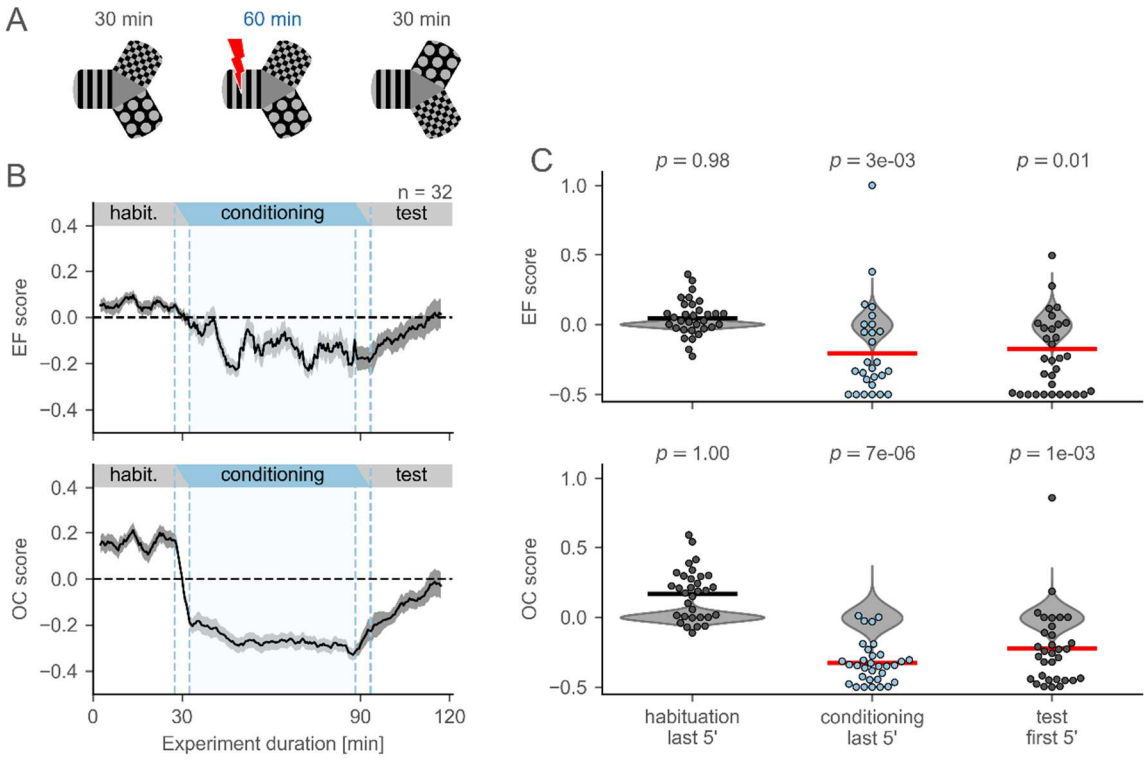

Figure S4. Zebrafish continue to avoid conditioned arm after swap of safe patterns. Related to Figure 6.

(A) Schematic of the protocol with the swap of the safe patterns in the test session. **(B)** EF (top) and OC (bottom) score moving averages. **(C)** Comparison of the EF (top) and OC (bottom) scores in the last 5 min of conditioning and in the first 5 min of the test session with the null-distribution (permutation test, n = 32 fish).

Table S1. Animals used in all experimental protocols. Related to Figure 1.

Experiment	Used in analysis	Excluded: overstayers	Excluded: unstable	Excluded: froze	Total	Age [dpf*]
Age comparison: 1 week	13	4	1	0	18	8
Age comparison: 2 weeks	10	7	0	0	17	14-16
Age comparison: 3 weeks	16	7	0	0	23	21-23
Control	24	--	--	--	24	22
Three patterns + test	40	13	6	4	63	21-25
Three patterns + no-patterns + test	30	9	1	0	40	20-24
Identical patterns	31	18	2	3	54	22-23
Swap of safe patterns	32	16	0	5	53	20-27
Replacement of shocked pattern	34	21	2	10	67	20-25
Control: pattern replacement	22	--	--	--	22	21-23
Rotation	31	14	3	8	56	21-23
Total	283	109	15	30	437	

Dpf – days post fertilization

Transparent Methods

Experimental model and subject details

Wild-type zebrafish of strain Tupfel Longfin (TL) were maintained at 28 degrees on a 14h-10h light-dark cycle. Embryos were obtained by spawning three adult fish pairs simultaneously. Embryos were raised in Danieau's buffer (17 mM NaCl, 2 mM KCl, 0.12 mM $MgSO_2$, 1.8 mM $Ca(NO_3)_2$, 1.5 mM HEPES) for the first 5 days of development. At 6 days post fertilization (dpf) the larvae were transferred to 3.5l tanks with fish system water (approx. 30 animals per tank). From 6 dpf to 20 dpf they were fed twice a day with live Rotifers and dry algae powder (Tetra Aufzuchtsfutter). From day 20 onwards, the diet was smoothly changed to a combination of freshly hatched artemia and Gemma micro dry food (Skretting). Animals were taken out of the fish facility and into the behavior room directly before the start of each experiment. Animals between the age of 8 and 27 dpf were used in the experiments. Males and females were not discriminated as the gonadal differentiation happens at a later stage of development. All animal procedures were conducted in accordance with the institutional guidelines of the Max Planck Society and the local government (Regierung von Oberbayern, animal license 55.2-1-54-2532-108-2016).

Experimental setup

The setup was custom-built in the lab. The walls and bottom of the Y-maze were laser-cut out of cast acrylic. The maze arms had a 1:1 width-to-length ratio, with a length of 30 mm; the walls were 10 mm high. Each arm opened to the triangular center of the maze, the ends of the arms were rounded. We used two identical mazes in parallel to allow the testing of two fish simultaneously, and therefore increasing the throughput. Walls of the two mazes were opaque to prevent fish from seeing each other during the experiment. Each maze had a piece of diffusive paper underneath for back projection of the visual stimuli (Rosco, Tough Rolux 3000). Both mazes and the diffusive paper were placed into a water basin, in order to remove the additional air layer between the screen and the fish and reduce light refraction. The scene was illuminated from below with a custom-built infra-red (IR) LED array to allow behavioral imaging. Both mazes were positioned under a high-speed camera (Ximea USB3.0 model MQ013MG-ON), in such a way that the walls did not produce a vertical shadow. The camera had a 6 mm lens (Thorlabs, Cat #MVL16M23) and an IR filter to filter out transmitted visible light. The setup was surrounded with black, opaque walls to shield the fish from distracting visual cues in the room.

Visual stimuli, designed in Python, were projected onto the diffusive paper with an LED-projector (LG model PA70G) via a cold mirror (Edmund Optics Cat # 64-452). The mirror was positioned at 45^o allowing IR light from below to pass through and light from the projector to be reflected onto the diffusive screen, as shown in Figure 1A. Stimuli were projected under the arms of the maze. The projected stimuli included (a) a pattern with black dots on light gray background; (b) a pattern with light gray dots on black background; (c) a pattern with black and light gray stripes; (d) a checkered pattern of black and light gray colors; (e) uniform gray (RGB = (128, 128, 128)). The light gray color was used instead of white to lower the brightness of the arena as high brightness could increase stress levels of the fish (personal observations). The patterns were designed such that the light-to-dark ratio was 1:1 in order to prevent differences in luminance between the stimuli, as larval zebrafish exhibit phototactic behavior (Burgess and Granato, 2007; Orger et al., 2000). The central area always had a uniform gray color (RGB = (135, 135, 135)), which was different from the gray in the arms to ensure that there was a contrast border at the entrance to the arms. Light gray RGB value was (180, 180, 180), black RGB value was (0, 0, 0).

Each arm contained a pair of electrodes located at the side walls. Each electrode was shaped as a 30-by-10 mm rectangle made out of steel mesh with wire diameter 0.2 mm, aperture 0.5 mm (Mijo Ilic Drahtgewebe-Shop.de) and covered the entire side wall. The electrodes were connected to a constant current stimulator (Digitimer DS3). Electrical stimulation was applied at 1 Hz in the periods between the entry and the exit of the fish from the conditioned arm. Electric pulses lasted between 50 and 100 ms, depending on the experiment (we did not observe differences in responses to shocks with these pulse lengths). Pulse amplitude was 0.7 mA (the value was chosen to elicit visible responses to the electric stimulus in all animals). The water used in the experiments was obtained from the fish facility (pH 7.5, temperature 28^{o}C, conductivity 650 µS).

Experimental protocols

Each experimental protocol consisted of one or more experimental sessions. The sessions followed each other without interruption, and each session could be characterized by its duration, the visual patterns that were projected onto the bottom of the Y-maze, and the ON/OFF status of the electric stimulation. In each protocol, every fish was tested individually and only once. 437 fish were tested in total.

Experiment was interrupted if a fish spent longer than one minute in the conditioned arm of the maze (equivalent to experiencing 60 shock pulses). This was done to prevent excessive stress for the animals. 109 fish were excluded from the analysis based on this criterion.

15 fish that did not reach a stable OC score (see "Measures for the CPA paradigm" below) at the end of conditioning were excluded from the analysis. The score was considered stable if the last point of the moving average lied within the 95th percentile range of the last 10 points of the moving average.

Finally, 30 fish that showed freezing behavior as a result of conditioning were also excluded from the analysis. Freezing behavior was identified by the 5th percentile of the distribution of total distance moved across all fish.

Overall, the analyzed dataset included 283 fish (see Table S1 for detailed numbers).

Behavioral tracking

All tracking was performed using custom-written code in Python, including the OpenCV library (Bradski, 2000). Black-and-white images were recorded at 60 fps with a resolution of 0.14mm per pixel. The position of the fish was identified in real-time using background subtraction. The background was calculated as a running average of the last 20 seconds of the recording. This time-dependent background was subtracted from the current frame, the result was filtered with a Gaussian filter with a 5x5 pixel kernel to remove point pixel noise, and then binary thresholded. The fish was identified as the contour with the largest area on the thresholded image. Fish position was calculated as the center of mass of the corresponding contour. The identified position was corrected using a Kalman filter to reduce the noise in the recordings (Kalman, 1960). Filter state variable included (x, y) position of the fish and x- and y-projections of the speed, and was updated for every frame of the recording using the observed fish position. Filter model for motion assumed movement with constant speed. Noise along x- and y-coordinates was assumed to be independent.

Swim bouts in Figure 1E were estimated from the time series of fish positions in the maze after the experiment by analyzing the recorded videos. First, speed of the fish was calculated as the Euclidean distance between positions at adjacent time frames. The speed was then filtered using a finite impulse response filter with a low-pass kernel (Parks–McClellan algorithm with 4Hz cutoff frequency) to remove high-frequency noise (Parks and McClellan, 1972). Swim bouts were defined as intervals of the filtered speed curve above a manually set threshold. The swim bout amplitude was calculated by integrating the area under the speed curve between the boundaries of the bout.

The fish's heading direction in Figure 4 was calculated for each frame of the recorded video. First, a contour of the fish was identified in a manner similar to the calculation during the experiment as described above. The terminal point of the heading vector was at the center of mass of the contour, which roughly corresponded to the head of the fish. The initial point of the vector was at the furthermost point of the contour from the center of mass, which corresponded to the tail tip of the fish. The heading direction was calculated as a relative angle between the heading vector and the horizontal edge of the image. Fish's orientation in the arm was calculated as a relative angle between the heading direction of the fish and the orientation of the arm. The orientation of the arm was given by the arm vector, whose initial point was at the maze center, and whose terminal point was at the arm center. Arm orientation was identified as an angle between the arm vector and the horizontal edge of the image. Fish's orientations in the arm were divided into four categories: "into the arm" for angles between -45^o and 45^o; "towards the anode" for angles between 45^o and 135^o; "out of the arm" for angles between 135^o and 225^o; "towards the cathode" for angles between 225^o and 315^o. "Towards the anode" and "towards the cathode" orientations were called aligned with the electric field, while "into the arm" and "out of the arm" orientations were called misaligned with the electric field.

Fish size was calculated as the Euclidean distance between the tip of the head and the tip of the tail, whose positions were manually picked by analyzing recorded videos. To reduce the assessment noise, the length was identified in five randomly picked frames of the video for each fish. Afterwards the final length was obtained by averaging the five handpicked lengths. The accuracy of this procedure was estimated with a coefficient of variation of fish size, calculated for every fish by dividing the standard deviation of manually measured fish lengths by the mean of those lengths. Coefficients of variation were calculated for a random sample of all experimentally tested fish (n = 42). The obtained values did not exceed 5%.

Measures for the CPA paradigm

Behavior in the CPA paradigm was assessed with two measures: occupancy of the arms (OC) and entry frequency of the arms (EF). OC of a particular arm, or maze center, was calculated as the proportion of time spent in the respective part of the maze. EF of a particular arm was calculated by dividing the number of times that the fish entered into that arm by the total amount of entries the fish performed into all arms. The two measures could be calculated for the whole time of an experiment as well as for a part of an experiment (in a corresponding time window).

We created a preference score in order to estimate learning effects. The score was calculated for each measure separately as the difference between the mean OC/EF of the conditioned arm and the average of OC/EF of the other two arms (Equation 1 for the occupancy score, OC_{score}, and Equation 2 for the entry frequency score, EF_{score}). Score values were above zero when the conditioned arm was preferred, and below zero when the arm was avoided. Note that the OC_{score} is calculated from the OC-values of the three arms, irrespective of the OC-value of the maze center.

$$OC_{score} = OC_{shock} - \frac{1}{2}\left(OC_{safe1} + OC_{safe2}\right) \quad (1)$$

$$EF_{score} = EF_{shock} - \frac{1}{2}\left(EF_{safe1} + EF_{safe2}\right) \quad (2)$$

The dynamics of the preference score throughout an experiment were visualized using moving average with a 5-minute time window and a 30-second step, i.e. two adjacent windows had a 4.5-minute overlap. Every point on the moving average represents the average OC/EF score across individual fish in a single time window. Error bars for the moving average correspond to the standard error of the mean. Some points of the moving average were calculated from the data of two adjacent sessions (sessions can have different experimental conditions, i.e. ON/OFF electric stimulation); vertical dashed lines mark intervals reflecting mixed experimental conditions.

As individual fish could have different number of arm entries within a 5-minute time window (e.g. because of different swimming speeds), we calculated the weighted average across individual fish for the EF score. The weights were proportional to the total number of entries for a particular fish in a particular time window.

Permutation test

Permutation test was used to assess the differences between the OC/EF measures in the conditioned arm and the other two arms of the maze. For each experimental protocol, a permutation test was performed for the last 5 minutes of the conditioning session (to estimate the effects of shocks during the conditioning) and for the first 5 minutes of the test session (to estimate the memory of the conditioning after the electric stimulation was switched off). OC/EF of each arm for each fish were calculated in these time windows. Then, for each fish separately, the arms were randomly relabeled, so that the OC/EF values were reassigned to different arms. Such relabeling (permutation) was performed $n = 10^6$ times. Preference score was then calculated for the experimental values and for each permutation as described above. OC/EF scores obtained from all permutations constitute a distribution of score values for the null hypothesis, i.e. that all arms are interchangeable for the fish, and therefore that there is no significant difference between the OC/EF of the conditioned arm and the other two arms. The experimental score value lies somewhere in this distribution. The significance of the experimental score value is assessed by calculating its position in the null-distribution (Equation 3).

$$p_{value} = P(Score^{null} \leq Score^{exp}) \qquad (3)$$

In the cases of very strong effects of conditioning sometimes none of the permutations produced a score lower than the experimental value. In such cases, the estimated *p*-value was equal to 0. In order to get an informative value, we fitted a Gaussian to the null-distribution, and calculated the probability of the experimental value to occur against the Gaussian. This Gaussian estimated *p*-value is reported for the experiments.

Clustering of shock-triggered swim bouts

The dataset for shock-triggered swim bouts in Figure 4 was obtained from the conditioning sessions of 27 fish. It contained responses to 14,519 shocks, each shock was 67 ms long. For every shock, fish coordinates were extracted for the 35-second interval starting at the shock onset. Every coordinate sequence was then transformed into frame-to-frame speed sequence, calculated from the Euclidean distances between coordinates from adjacent time frames. Every speed curve was smoothed using univariate splines. Speeds whose peak values were lower than a threshold value of 21.4 mm/s were considered non-responses (n = 10,428); the rest were considered swim bouts (n = 4,091). This threshold was chosen such that automatically and visually identified swim bouts matched (data not shown). Additionally, we calculated the orientation of the fish in the conditioned arm at the onset of each identified swim bout.

Principal-component analysis was performed on the dataset of smoothed bout speed curves in order to identify their important features (Jolliffe, 2002). Four principal components could explain more than 90% of total variance in the dataset, and were selected as the main features of the speed curves. Hierarchical clustering with Ward's linkage method was performed on a bout dataset, which was represented by a 4,091-by-4 matrix (4 principal components per bout, orientation in the arm was not included as a parameter for the clustering). Ward's linkage method minimizes the sum-of-squares within the clusters (Ward, 1963). The cut level for the cluster tree was chosen so that three clusters emerge. Every cluster was considered to represent a separate type of response to shocks.

Comparison of avoidance levels between different response types was performed using the one-way Kruskal-Wallis test, followed by a post-hoc Mann-Whitney test for group comparisons. Three response types were compared against each other (3 pairs). The avoidance levels were estimated by calculating the OC/EF scores in the next 5 minutes after every individual shock response of a particular type.

Separation of avoidance strategies

Avoidance strategies in Figure 5 and Figure 6 were estimated using hierarchical clustering with Ward's linkage of the arm occupancies in the last 5 minutes of conditioning. For each fish, we computed the occupancy of each arm as described above and re-centered them around zero (so that they sum up to zero). Negative arm occupancy means avoidance, and positive arm occupancy corresponds to preference of the arm. Additionally, we calculated the occupancy of the center. As a result, for each fish a vector of 4 values was calculated: 3 corrected arm occupancies and 1 center occupancy. Importantly, the three occupancies for the arms were linearly dependent, so we excluded one of the arms in clustering. In total, clustering was applied to a 179x3 occupancy matrix, where 179 rows corresponded to individual fish (note that we used fish pooled from different experiments with identical conditioning sessions). Before clustering, values within each column were standardized by centering around the mean and scaling to unit variance.

At each node of the clustering dendrogram, we estimated the significance of splitting the subtree under that node into two clusters. For any current subtree we permuted occupancy values within the columns of the corresponding occupancy matrix (10^4 permutations in total). We used the same clustering method to get two clusters for each permutation, and obtained linkage values between these two clusters. These linkage values formed a null-distribution corresponding to the hypothesis that there is no separation of the subtree at the investigated node. Then, the position of the original, non-permuted, linkage value was identified in the null-distribution. The significance threshold was chosen to accommodate multiple comparisons between different subtrees of one dendrogram. Briefly, for the node J the significance value

p^J_{value} was adjusted by dividing the chosen significance level (0.05) by the number of nodes between the current node and the root of the dendrogram, D_J (Equation 4).

$$p^J_{value} = p_{value} \cdot \frac{1}{D_J} \quad (4)$$

Statistical significance of cluster separation was estimated recursively, starting from the root and investigating each of the following two subtrees. Using this method, we identified six statistically significant clusters. For visualization and explanation purposes, we combined four clusters with the lowest occupancy of the conditioned arm into the 'arm-avoiding' group, while the other two clusters corresponded to 'non-avoiding' and 'center-preferring' groups (see Figures 5 and 6).

Memory-less model

A pseudo-random walk model in Figure 1 was designed to investigate how reactions to shocks, independent of learning, could influence the OC and EF measures of the conditioned arm. In the model, the arms of the Y-maze were reduced to three one-dimensional (1D) linear tracks. Each 1D arm had a length (parameter *L*) and a coordinate axis associated with it, with the arm opening located at 0, and the arm end located at a distance *L* from the origin. The center of the maze was modeled as a separate 1D compartment of length L_{center}. The simulated agent moved along the arm axis with discrete steps (bouts). Each step had a direction (towards the left or the right boundary of the arm) and a size *S*. In order to choose the step size *S*, we used the experimentally observed distribution of swim bout amplitudes (Figure 1E, gray). The experimental distribution was fitted with a Gamma distribution (equation 5) with shape parameter $k = 1.958$ and scale parameter $\theta = 0.999$ mm.

$$f(x,k,\theta) = \frac{x^{k-1}\exp(-\frac{x}{\theta})}{\theta^k \Gamma(k)}, \qquad \text{where} \quad \Gamma(z) = \int_0^\infty x^{z-1}e^{-x}dx \quad (5)$$

The mean of the fitted distribution, calculated as a product of shape and scale parameters, was equal to 1.956 mm. Thus the average swim bout amplitude, relative to the maze arm length (30 mm), was 1.956 mm/30 mm = 0.065. In order to match these parameters in the model, we drew the step size *S* from a Gamma distribution with mean of $0.065 \cdot L$, where *L* was the length of the arm in the modeled maze. Similarly, in terms of *L*, the scale parameter θ can be written as $\theta = 0.999 \text{ mm} \cdot L/30 \text{ mm} = 0.033 \cdot L$.

If the simulated agent moved beyond the left arm boundary, it exited its current arm and entered the central compartment. When the simulated agent moved to the right arm boundary, it stopped there until the next step of the simulation ('sticky' boundary conditions). Both boundaries of the central compartment were treated equally: if the simulated agent stepped over either the left or the right boundary of the central compartment, it entered an arm. Each arm had a probability of entry associated with it, all probabilities summing to one. The effects of the electric shocks could be simulated in one of the arms. The size of every step made in the shocked arm was multiplied by a parameter $\alpha \geq 1$, to simulate the increased swimming speed in response to electric shocks.

The model without learning was used to investigate if the increased speed in the conditioned arm alone could explain the changes in OC/EF measures during conditioning. An experiment was simulated with 3 sessions. Each session lasted *n* steps. In the starting habituation session, the step sizes *S* of the simulated agent in all arms were drawn from the same Gamma distribution. At the end of the habituation session, the arm with the highest occupancy was selected as the conditioned arm for the following conditioning session (occupancy was higher in one arm due to stochastic reasons). In the conditioning session, the sampled step size *S* of the simulated agent in the conditioned arm was multiplied by the parameter $\alpha > 1$ to simulate increased speed during the shocks. In the third (test) session, step sizes in all of the arms were again drawn from the same distribution. Probabilities of entry into any of the arms were equal to 1/3 in all sessions. All simulations were run using custom Python code. Parameters used for simulations in Fig 1: $L = 5$; $L_{\text{center}} = 1.5$; $k = 1.96$, $\theta = 0.033 \cdot 5 = 0.165$; $\alpha = \{1, 2, 3, 4\}$; $n_{\text{habituation}} = 20{,}000$ steps; $n_{\text{conditioning}} = 40{,}000$ steps; $n_{\text{test}} = 20{,}000$ steps.

A learning component was added to the model to investigate if the learning measures can be used to detect learning effects. In the model with learning, the probability of entry into the arms of the simulated

maze was changed from a constant to a variable parameter. Under the learning rule, each entry into the conditioned arm during conditioning decreased the probability of returning to that arm. This rule corresponded to an exponential decay of the probability of entry, with a learning rate β and a floor of 0.1 (a non-zero value was chosen because in the experiments the probability of entry never reduced to 0, Equation 6). The probabilities of entry into the other two arms increased correspondingly, to keep the sum of all probabilities equal to one (Equations 7 and 8).

$$\Delta p_{entry}^{shock} = \beta \cdot \left(0.1 - p_{entry}^{shock}\right) \quad (6)$$

$$\Delta p_{entry}^{non-shock} = -\frac{1}{2} \cdot \Delta p_{entry}^{shock} \quad (7)$$

$$\sum_{all\ arms} p_{entry} = 1 \quad (8)$$

Memory extinction in the test session was simulated by slowly letting the probability of entry into the conditioned arm rise back to the 1/3 level (Equation 9). The update of the probability occurred at every return to the previously conditioned arm. The probabilities of entry into the other two arms were decreased correspondingly (Equation 10 and 11)

$$\Delta p_{entry}^{shock} = \beta \cdot \left(0.33 - p_{entry}^{shock}\right) \quad (9)$$

$$\Delta p_{entry}^{non-shock} = -\frac{1}{2} \cdot \Delta p_{entry}^{shock} \quad (10)$$

$$\sum_{all\ arms} p_{entry} = 1 \quad (11)$$

The learning rate β used in Figure 1 was equal to 0.03.

Supplemental references

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