

Long-Term Social Recognition Memory in Zebrafish

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Abstract

In species in which individuals live in stable social groups, individual recognition is expected to evolve to allow individuals to remember past interactions with different individuals and adjust future behavior toward them accordingly. Thus, social memory is expected to be a ubiquitous component of social cognition of social species. However, few studies have investigated the occurrence of social memory in non-mammals. Here we evaluated the ability of zebrafish (*Danio rerio*) to recognize different conspecifics and to retain this information in long lasting (i.e. 24 h) memories. We used a social discrimination paradigm, adapted from mouse studies, in which the focal individual meets two pairs of conspecifics in two consecutive days: one conspecific is the same in both days and the other is different between days 1 and 2. If animals have the ability to discriminate between different conspecifics, it is predicted that they will spend more time exploring the novel than the familiar (i.e. already seen in day 1) conspecific. In this study, zebrafish with access to both olfactory and visual conspecific cues exhibited consistent recognition of a previously encountered (familiar) conspecific after a 24 h delay. This result supports the hypothesis that long-term social memory, previously described in mammals, is also present in zebrafish, hence extending the evidence for the presence of this type of memory to teleost fish.

Keywords: social recognition, memory, zebrafish

Introduction

GROUP-LIVING ANIMALS INTERACT frequently with other conspecifics and their ability to adjust the expression of behavior to optimize their social interactions (also known as social competence) has a major impact on Darwinian fitness.¹ A key component of social competence in species in which repeated interactions among the same individuals occur, such as in the case of stable social groups or pair bonding, is the ability of individuals to recognize other individuals, so that they can remember past interactions with them and adjust future behavior toward them accordingly.² This ability is critical for territorial defense, establishment of dominance hierarchies, pair bonding, and mating (reviewed in Refs.^{3,4}). Importantly, social recognition does not necessarily require individual recognition because animals may categorize, and therefore recognize, individuals according to different social categories, such as species, group member, kin, age, sex, reproductive status, and/or hierarchical status.⁴ Thus social recognition can be defined as the ability to categorize behavioral agents into different classes and to recall the learned idiosyncratic identity of a specific individual previously met.⁴

At the cognitive level, social recognition relies on recognition memory, which is the ability of individuals to discriminate between familiar and unfamiliar stimuli, based on the storage of a memory during an initial encounter that is retrieved during posterior encounters with those stimuli.⁴ Recognition memory is not specific to social stimuli and has been described toward objects (object recognition,^{5,6}). Social recognition memory has been mainly studied in rodents, whereby two types can be identified based on their temporal persistence: short-term memory, with a limited duration minute to a few hours,⁷ and long-term memory with durations from 24 h up to 7 days.^{8,9}

In laboratory conditions, social memory can be evaluated by changes of spontaneous exploratory behaviors directed toward conspecifics when an individual is re-exposed to a familiar or a novel conspecific. The discrimination procedure is a commonly used behavioral test to study social memory in laboratory animals, which was first described by Engelmann *et al.* It is based on a binary choice test between a novel and a familiar conspecific.¹⁰ Similar to other nonsocial cognitive tests (object recognition), this paradigm allows the assessment, within the same test, of an animal's discrimination between two social stimuli. In this test, social recognition is

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assessed by comparing the difference in the time spent investigating the familiar versus the unfamiliar conspecific.

In this article, we have investigated the occurrence of social memory in zebrafish (*Danio rerio*). In the past decades, zebrafish has become a major model organism in neuroscience, because of the tools available for neural circuit observation and manipulation (e.g., whole brain/single cell functional imaging,^{11,12} optogenetics,^{13,14} and *in vivo* synaptic activity,¹⁵ and the similarities in brain function with other vertebrates.¹⁶ Thus, zebrafish have been used in translational studies of attention,¹⁷ memory,^{18–20} learning,^{21–23} anxiety,²⁴ addiction,^{25,26} and stress.²⁷ In the field of memory, several studies clearly demonstrate the mnemonic abilities of zebrafish, such as olfactory conditioning,²⁸ shuttle box learning,²⁹ appetitive choice discrimination,³⁰ Y-maze memory task,¹⁹ aversive reinforcement learning,³¹ classical and operant conditioning,^{32,33} inhibitory avoidance,¹⁸ and conditioned place preference.³⁴ Moreover, zebrafish are highly social, living in aggregations (also known as shoals³⁵) that offer ecological benefits such as reduction of predation risk and enhancement of foraging and reproductive success.³⁶ Interestingly, zebrafish do not associate randomly, rather they show visually mediated preferences for fish of a phenotype similar to that of their rearing companions.³⁷ Hence, interactions early in life shape subsequent shoaling choices, suggesting the occurrence of a discrimination process based on recognition. This ability seems to be based on olfactory and visual imprinting that occurs early in development.^{38,39} Research on visual cues suggests that zebrafish prefer to shoal with conspecifics with same size and coloration pattern and that females can distinguish males based on visual cues alone.^{40–42} Olfactory cues have also been shown to play a role in shoal preferences, and kin recognition based on olfactory cues has also been described.⁴³ Thus, zebrafish have the ability to use both visual and chemical cues to base their social preferences. Moreover, stable dominance hierarchies over time are present in zebrafish shoals.³⁵ Together, all these pieces of evidence suggest the occurrence of social memory in zebrafish.

The main goal of this study is to test whether long-term social recognition memory (i.e. 24 h recall) is present in zebrafish. We used the social discrimination paradigm,¹⁰ rather than the habituation–dishabituation paradigm,⁷ because in mice the former has proven to be more robust.^{10,44}

Materials and Methods

Ethics statement

The animal experimentation procedures used in this study followed the institutional guidelines for the use of animals in experimentation and were approved by the internal Ethics Committee of the Gulbenkian Institute of Science and by the National Veterinary Authority (DGAV, Portugal; permit number 0421/000/000/2013).

Animals and housing conditions

Adult [i.e. 7–10 months old, weight = 0.35 ± 0.05 g (mean \pm standard error of the mean [SEM]), standard length = 3.7 ± 0.17 cm (mean \pm SEM)] zebrafish males ($N=41$) from the Tubingen (Tu) wild-type strain were used as focal fish. Fish were housed in groups of eight individuals per tank ($40 \times 20 \times 30$ cm) at 28°C in a 14 h light: 10 h dark cycle. Water quality was monitored weekly for nitrites (<0.2 ppm), nitrates

(<50 ppm), and ammonia (0.01–0.1 ppm); pH and conductivity were kept at 7 and 700 μ Sm, respectively. Fish were fed twice a day with GEMMA 300 and *Artemia salina*. Adult males of the same strain, but from different crosses (i.e., with different genetic background), were used as social stimuli. Focal and stimuli fish were reared and housed separately to avoid any familiarity effect.

Animal tagging

A week before the start of the experiment, fish were anesthetized with tricaine solution (MS-222, Pharmaq; 320 mg/L), weighed, measured for standard length, and individually tagged with a monofilament line painted with nail polish of different colors.⁴⁵ Animals recovered from the tagging procedure in individual tanks for 30 min and then were transferred back to their group tanks.

Social memory test

A social discrimination paradigm was used to evaluate social memory and determine whether a zebrafish can distinguish between a familiar and a novel conspecific. This paradigm exploits the natural preference of zebrafish for novelty⁴⁶ and was adapted from mouse studies.¹⁰ Behavioral tests were run in a $60 \times 20 \times 20$ cm glass tank, divided, by a removable transparent partition, into three equally sized compartments (Fig. 1A). The walls of the experimental tank were covered with a white plastic sheet, so that no stimuli from outside the tank could be seen during the experiment.

The behavioral task comprised a habituation, acquisition, and a test phase separated from each other by 24 h (Fig. 1B).

Apparatus habituation. Before the start of the experiment, focal fish were isolated overnight in individual tanks ($15 \times 20 \times 20$ cm) to control for putative effects of previous social status on their performance in the test. On day 0 of the experiment, each focal fish was placed in the central compartment of the experimental tank and allowed to become familiar with the empty tank for 5 min. At the end, focal fish were captured and returned to their individual tank.

Acquisition phase. In the memory acquisition phase (day 1), each focal fish was placed in the central compartment and allowed to explore it for 5 min. Then, the partitions were removed and the focal fish were allowed to explore the other two compartments, each containing a nonfamiliar conspecific male, for 20 min. The prediction is that because both stimuli fish are new to the focal fish, it will explore each of them for similar amounts of time. After this period, the animals were caught and returned to individual tanks.

Test phase. The test phase (day 2) followed exactly the same protocol of the acquisition phase, but in this phase, the focal fish was exposed to one of the fish it had seen in the acquisition phase in the one compartment versus a new nonfamiliar fish in the other compartment. The prediction is that if the focal fish can discriminate between the previously observed stimulus fish and the newly introduced fish, it should spend more time with the latter because of novelty preference.

Stimuli fish (familiar and nonfamiliar) were presented to the focal fish inside transparent acrylic parallelepipeds

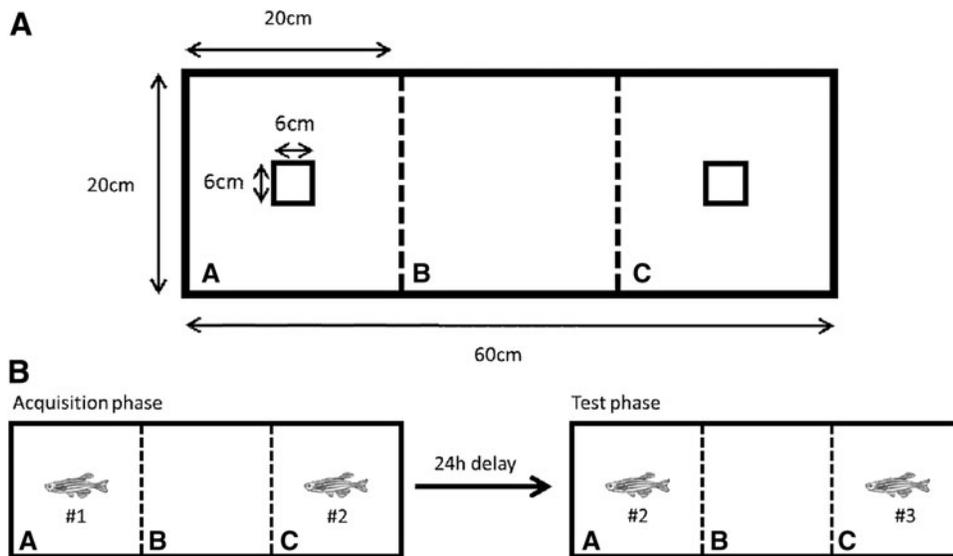


FIG. 1. Schematic representation of the behavioral setup. **(A)** Top view of experimental setup. The *dotted lines* represent removable partitions and the *squares* represent the localization of stimuli fish. **(B)** Diagram of the recognition memory paradigm. **A** and **C** represent the compartments where the stimuli fish were presented; **B** represents the starting (neutral) compartment.

(6×6×20 cm) that were placed inside each of the lateral compartments of the experimental tank. These parallelepipeds were perforated (120 holes with 1 mm of diameter each). We have checked whether a chemical signal present inside the parallelepipeds could reach the outside by injecting 500 μL of toluidine blue stain inside the parallelepiped and checking whether it would appear in the outside compartment. In stirred water (which simulates the presence of a swimming fish), it took 3 s for the stain to appear outside. Hence they allowed visual, olfactory, and acoustic interaction between the focal and the stimuli fish. Before the start of the experiment, all stimuli fish were habituated to stay inside the parallelepipeds, and during the experiment they showed no signs of distress from being in it.

Behavior analysis

An overhead video camera was used to record the focal fish behavior during the test, which was subsequently analyzed using a video-tracking system (Ethovision[®] XT8, Noldus, The Netherlands). The time spent by the focal fish in each of the three compartments (A, B, and C, Fig. 1A) was recorded in both days (days 1 and 2). A preference for the novel fish in day 2 (i.e., more time spent exploring it) would be indicative of social recognition, indicating that the fish is able to discriminate a previously seen and a novel individual. We have also used a measure of social motivation expressed as a social exploration score: $\text{social exploration score} = \left(\frac{\text{time in A} + \text{time in C}}{\text{time in A} + \text{time in C} + 2(\text{time in B})} \right) \times 100$. This index can vary between 0% and 100% of time, and values >50% reveal a preference to associate with conspecifics.

Statistics analysis

To check the assumptions of parametric statistics, we checked all data for normality using the Shapiro Wilk's W test and evaluated skewness and kurtosis values. Homoscedastic was also tested using Levene's test.

Comparisons between the social investigation time obtained for day 1 (i.e., novel fish 1 vs. novel fish 2) and for day 2 (e.g., novel vs. familiar fish) were performed using a two-

tailed Student's *t*-test. The exploration scores obtained for day 1 were compared with those for day 2 using a two-tailed Student's *t*-test. Effect sizes were computed for each test using Cohen's *d*; following Cohen's proposal, *d*=0.2 indicates a "small" effect size, 0.5 represents a "medium" effect size, and 0.8 represents a "large" effect size. All data are represented as mean \pm SEM. Statistical significance was set at $p < 0.05$.

Results

Zebrafish discriminated between a familiar and a novel conspecific 24 h after the first exposure to the familiar conspecific. As predicted, on day 1, focal fish showed no preference between two unfamiliar individuals (day 1–novel 1: 519.9 ± 30.81 s; novel 2: 546.7 ± 26.41 s; $t(40) = -0.5479$; $p = 0.59$, Cohen's *d* = 0.14; Fig. 2A), but on day 2 it showed a preference for the novel fish (day 2–familiar: 434.3 ± 24.56 s; novel: 509.0 ± 26.77 s; $t(40) = -2.0319$; $p = 0.048$, Cohen's *d* = 0.45; Fig. 2A). Zebrafish explored significantly more conspecifics on the first exposure than on the second exposure (day 1: $72.58 \pm 1.73\%$; day 2: $62.32 \pm 2.0\%$; $t(40) = 6.791$; $p = 0.000$, Cohen's *d* = 0.85; Fig. 2B).

Discussion

Performance on social recognition memory requires the ability to identify and remember information about individuals. In rodents, this information can be stored for up to 60 min (short-term memory), or maintained for longer periods of time (24 h⁴⁷; 7 days⁸), reflecting long-term memory. In this study, zebrafish exhibited consistent recognition of a previously encountered (familiar) conspecific after a 24-h delay. This result supports the hypothesis that long-term social memory, previously described in mouse models,⁸ is also present in zebrafish, hence extending the evidence for the presence of this type of memory to teleost fish. Thus, social recognition memory seems to be an evolutionary conserved mechanism among social vertebrates, whereby individuals experience repeated interactions among themselves. The ubiquity of social recognition memory across vertebrates may

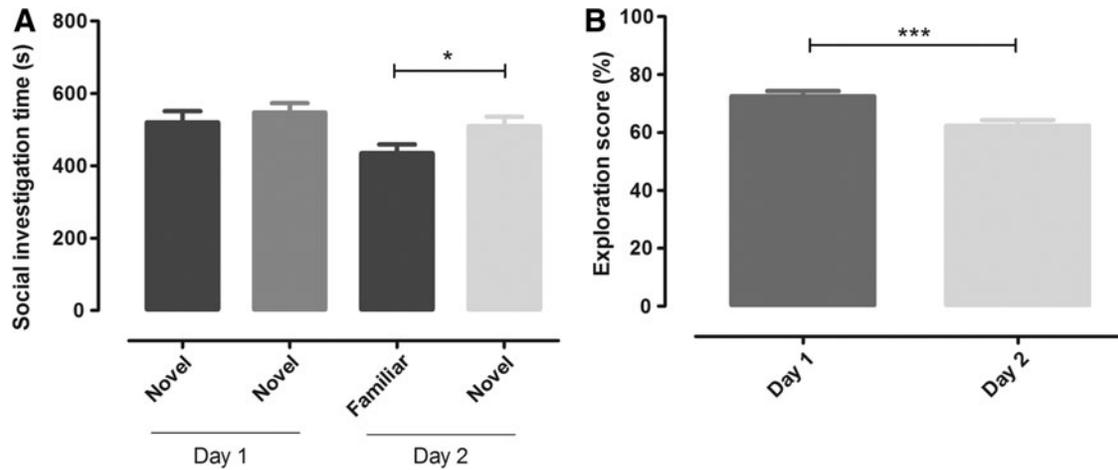


FIG. 2. Social recognition memory and stimulus exploration in zebrafish. **(A)** Zebrafish discriminate familiar from nonfamiliar conspecifics 24 h after the first encounter. **(B)** On the second day of test, individuals reduce significantly the exploration time, $*p \leq 0.05$; $***p \leq 0.001$.

be explained by its core adaptive value in species with repeated social interactions, because it allows individuals to adjust their behavior according to the individual they are interacting with based on past experience. This is a critical ability to manage social interactions in social hierarchies,^{2,48,49} to preferentially use different conspecifics as sources of social information (e.g.⁵⁰), or to adjust cooperative behavior based on familiarity (e.g., cleaner fish⁵¹).

Since the main aim of this study was to investigate the presence of social recognition memory in adult zebrafish, and given the published results on both chemical and visual imprinting of young zebrafish on conspecifics with which they are raised,³⁹ individuals were tested with both visual and chemical access to conspecifics. Thus, it is not possible to disentangle the relative contribution of each of the sensory modalities to the observed social recognition memory, but both are expected to have contributed significantly. Chemical signaling is essential in the aquatic environment, and the use of chemical cues may be particularly useful when visual communication is limited (e.g., darkness, turbid water, and highly structured environments). Chemical communication is widely used in fish to convey information about age, sex, and dominance status that mediates social interactions, including mate choice and dominance relationships.^{52–54} A general form of recognition based on odor cues has been documented in fathead minnows (*Pimephales promelas*) and sticklebacks (*Gasterosteus aculeatus*),⁵⁵ and in zebrafish, chemical cues play a critical role in phenotype matching processes.³⁸ In contrast, visual information can be updated more rapidly and allow for more fine-grained discrimination. In guppies (*Poecilia reticulata*), the recognition of particular individuals seems to be based on a more specific type of recognition that requires the use of visual cues,⁵⁶ and in zebrafish, visual imprinting on the coloration pattern of conspecifics has been described.^{42,40} Thus, social recognition in zebrafish may occur independently on each sensory modality or they may interact. In fish, the terminal nerve (also known as *nucleus olfactoretinalis*) is located in the olfactory bulbs and projects to the telencephalon, the tectum, and to the contralateral retina.⁵⁷ This pathway receives synaptic inputs from the olfactory bulbs and seems to be involved in processing sensory

information of other modalities such as visual and somatosensory information.⁵⁸ Thus, rather than being a sensory nerve, the terminal nerve seems to be involved in olfactory modulation⁵⁷ and the integration of the two sensory systems may be relevant for social recognition in zebrafish.

Our results also show that individuals explore more new individuals rather than familiar individuals. This result can be viewed as unexpected, because zebrafish form structured shoals and prefer to shoal with familiar relatives.³⁷ However, shoaling preferences are based on learned social preferences such as appearance, kinship, or current behavior of individuals^{37,59} and should be distinguished from familiarity preferences. In some cases, fish could avoid familiar individuals instead preferring unfamiliar individuals,⁴⁶ a preference that maximizes mating opportunities for males.

In summary, our findings suggest that in zebrafish, social recognition memory may be achieved using a combination of visual and chemical cues that are used as self-referent matching, but future studies are needed to clarify the role of each sensory modality.

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Disclosure Statement

No competing financial interests exist.

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