Development of social behaviour in young zebrafish.

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Abstract

Adult zebrafish are robustly social animals whereas larvae are not. We designed an assay to determine at what stage in development zebrafish begin to prefer and interact with conspecifics. We find a very weak attraction to conspecifics for one week old fish, however most three week old zebrafish, given a choice, strongly prefer to remain in the compartment where they can view conspecifics. However, for some individuals, the presence of conspecifics drives avoidance instead of attraction. This social preference is dependent on vision and requires viewing fish of a similar age/size. In addition, fish also gradually increase the degree of social interaction, measured as a tendency to coordinate their movements. Finally, social preference and interaction are differentially modified by an NMDAR antagonist and acute exposure to ethanol, both of which are known to alter social behaviour in adult zebrafish.

Introduction

Human infants exhibit social behaviours from birth (Xiao et al., 2014). Throughout life these innate social drives provide the substrate for learning more complex forms of human interaction. Disruptions to early social behaviour may impair the development of normal adult sociality, and may contribute to disorders such as autism (Banerjee et al., 2014). Since the neural circuitry that underlies human innate social behaviour is established *in utero*, very little is understood about its normal and pathological development, anatomy, and function.

Early developing social behaviours, such as the preference to observe and mimic conspecifics, are common to many other mammals (Ferrari et al., 2006) and lower vertebrates (Engeszer et al., 2004; 2007; Mooney, 2014). Animal models are much more amenable to detailed investigation and share many of the same anatomical and functional neural systems that underlie innate social behaviour in humans (O'Connell and Hofmann, 2011; 2012). Consequently, we sought a model system for which neural circuits can be assessed throughout development, and for which social behaviour is an important component of the organism's behavioural repertoire (Oliveira, 2013).

Zebrafish adults are social animals (Oliveira, 2013), exhibiting a range of group (shoaling and schooling; (Krause et al., 2000; Green et al., 2012; Miller and Gerlai, 2012), conspecific directed aggression (Jones and Norton, 2014), mating (Engeszer et al., 2008) and other behaviours (Arganda et al., 2012). Larval zebrafish, however, do not exhibit the overt shoaling and schooling behaviours that are readily apparent in adults. In order to shoal, fish must decide to approach and remain near conspecifics, and there is some evidence that such a preference might appear as early

as one week (Hinz et al., 2013), whereas shoaling is appears in post flexion larvae (~three weeks old, 7 mm length) (Engeszer et al., 2007).

Social behaviour encompasses more than simply preferring to be near members of the same species. For instance, individuals may coordinate their behaviour with other members of the same social group. Such coordination is obvious in the case of schooling fish, where individuals align their body orientation and synchronize their movements, but it is also present in social mammals. For example, humans will unconsciously coordinate a diverse range of behaviours, such as yawning, eye blinks and posture (Sebanz et al., 2006; Richardson et al., 2007), and this is thought to provide a foundation for more elaborate forms of social communication and cooperation.

Here we set out to investigate early social interactions in zebrafish and to determine if the establishment of preference for the presence of conspecifics is contemporaneous with individuals beginning to coordinate their behaviour. We have designed a novel social preference/interaction assay for zebrafish larvae that continuously monitors the detailed behaviour of individual zebrafish freely choosing to visually observe or avoid a counterpart. This assay demonstrates that social behaviours develop gradually and are robust by three weeks post-fertilisation. We have also used the assay to characterize the effects of substances known to influence the social behaviour of adults.

Results

Fish develop strong social preference and interactions by three weeks of age

We designed a behavioural chamber in which zebrafish fry could swim freely between two arms, but in only one could they view conspecific siblings through a glass partition. Six such chambers were simultaneously monitored with an infrared high-speed camera and automated tracking software recorded the behaviour (position and orientation) of the observer fish (Fig. 1a, Supp. Movie 1). Following 15 minutes in the chamber without conspecifics (acclimation (A) period), a single or three conspecifics were added to one of the adjacent compartments, randomly selected, and the behaviour of the observer fish was monitored for an additional 15 minutes (social cue (SC) period). There was no bias between compartment arms in the acclimation phase for fish at any age, nor if the fish were monitored for a further 15 minutes following the acclimation phase without adding the SC (Supp. Figure 1c).

Three-week old zebrafish consistently showed a very strong bias to remain in the arm of the chamber adjacent to the SC (Fig. 1b, Supp. Movie 2, 3). To quantify the tendency for each tested fish to spend time in one or the other arm of the chamber, we defined a social preference index (SPI) (see Methods). A positive SPI indicates a preference for the chamber arm with the SC and a negative SPI indicates an aversion for the SC. The SPI was computed for all tested one, two and three-week old fish with and without the presence of multiple conspecifics (Fig 1d). One-week old larvae exhibited a weak preference for the SC arm (Fig. 1d, top; A vs SC t-test, $p=0.09$). The SPI of two week old larvae was shifted towards positive values (Fig. 1d, middle; A vs SC t-test, $p= 3.4*10^{-6}$) and by three weeks, the population was strongly positive with many values close to 1, reflecting the strong bias of many observer fish to remain almost entirely on the side of the conspecifics (Fig. 1d, bottom; A vs SC t-test, $p=1.2*10^{-14}$). Social preference did not depend on having multiple conspecifics in the social viewing arm as a similar pattern of SPIs was observed when only a single

conspecific served as the SC (Fig 1e, Supp. Fig. 1b; A vs SC phases t-test, p=0.9 for one week; $p=4.9*10^{-8}$ for two weeks, $p=2.9*10^{-16}$ for three weeks).

A small minority of three-week old fish had strong negative SPIs (Fig 1d, bottom). These fish exhibited an aversive bias to the conspecifics, preferring to stay in the opposite chamber away from the SC (Supp. Fig. 1a and Supp. Movie 4). Such aversive behaviour was rarely observed in younger fish suggesting that as for positive social interaction, this aversive behaviour develops to become more frequent and robust over time.

The behaviour of the three-week old zebrafish when viewing the SC consisted of alternating body orientation such that the left or right eye directly viewed the SC compartment (Supp. Movie 3). This behaviour was quantified in a histogram of all orientations of the fish body axis while in the SC arm of the chamber (Fig. 1c). A gradual transition from primarily orienting along the axes of the chamber (cardinal directions: 0°, +/- 90°, and 180°) to orienting for visual observation (+/- 45°) occurred over the first three weeks of development. No strong bias for observing with either the left or right eye was found in this assay (Sovrano and Andrew, 2006).

We next set out to investigate what sensory cues contribute to the displayed social preference.

Social preference requires visual observation of conspecifics of a similar age

Although visual stimulation seemed the most likely source of the preference for the SC, it was possible that some olfactory or tactile cues may pass between the chambers of the observer and SC fish. Consequently, we compared preference behaviour for three-week old fish tested in the dark to those tested in light (Fig. 2a).

Removal of background illumination completely abolished the tendency of observer fish to orient towards the conspecific viewing chamber (Fig. 2b) and social preference was abolished, as evidenced by the distribution of SPIs (Fig. 2c, and Supp. Fig. 2a; A vs SC t-test, p=0.5). These experiments provide strong evidence that the social preference behaviour of three-week old zebrafish depends on vision.

The data above indicates that during the first three weeks of their life, zebrafish develop a robust social preference to view age-matched conspecifics. However, during this time they also change significantly in size, doubling their head to tail length (Supp. Fig. 2b). To assay whether the size/age of the SC fish influences social preference, we monitored the behaviour of one and three week old zebrafish presented with larger/older or smaller/younger conspecifics as the SC (Fig. 2d and Supp. Fig 2c).

One-week old fish showed no significant preference for three week old fish, supporting the conclusion that the development of social preference reflects maturation of the observer and is not simply dependent on the age/size of the stimulus (Fig. 2d left; A vs SC t-test, p=0.015). Three-week old fish also did not exhibit a strong social preference when presented with one-week old fish as the SC (Fig. 2e, right; A vs SC t-test, p=0.07). However, the broader distribution of SPIs suggests that the smaller, younger fry did influence the behaviour of the larger, older observing fish. This may be due to fish becoming progressively more attentive to any moving objects within their environment.

Zebrafish fry coordinate their movement

Three-week old fish display robust visually-driven social preference; high-speed videography additionally allowed us to investigate the extent to which the behaviour of the SC fish influenced the behaviour of the observer.

Young zebrafish tend to move in small bouts of activity consisting of discrete swims or turns (Orger et al., 2008). Individual bouts were detected by identifying a peak in the motion tracking signal (Fig. 3a, top trace). Averaging all of these bout time-courses (Fig. 3b) revealed a pre- and post-bout quiescent period, the timings of which reflected the periodicity of movement. These quiescent periods shortened from one to three weeks of age as the mean bout frequency increased ~50%, from 0.79 Hz at one week to 1.22 Hz at three weeks. As observed in other behavioural contexts, these movement bouts were composed of a mixture of forward swims and orienting turns (Fig. 3c) (Orger et al., 2008).

We next asked whether a motion bout produced by the SC fish influenced the movement of the observer. Short time windows of the motion trajectories from the observer fish, normalized by each individual's average motion peak, were extracted and aligned to the bouts of the SC fish (Fig. 3a, middle trace) and were then averaged over all bouts (Fig. 3a, bottom trace). This generated a 'bout triggered average' (BTA), which is an estimate of how the motion bout of the SC influences movement of the observer.

A clear interaction between the movement of the SC fish and the observer was present at all stages of development. Notably, a bout of motion by a SC fish was, on average, coincident with a synchronous increase in motion by the observer fish. The strength of this motion coupling increased substantially over development (Fig. 3d), correlating with the enhancement in positive social preference. This visual coupling behaviour was, unsurprisingly, absent for fish in the dark (Fig. 3e). These results indicate that not only do three-week old fish prefer to be with conspecifics but that their behaviour is coupled with that of their social partners.

Social preference and interaction are differentially impaired by drug exposure

We next assayed whether pharmacological manipulations that affect sociality in adult animals similarly influence the manifestation of social behaviour in young zebrafish.

Social learning in adult zebrafish is dependent upon N-methyl-D-Aspartate (NMDA) Receptor signalling(Maaswinkel et al., 2013) and we first assessed whether manipulating this pathway altered the social preference and interaction behaviour of fry. The NMDA receptor antagonist MK-801 was acutely administered at a concentration of 100 μm for 1 hour prior to assaying three-week old zebrafish (Fig. 4a-d). Although MK-801 can lead to increased locomotor activity(Menezes et al., 2015), at this concentration and age treated zebrafish showed no significant change in the overall amount of swimming activity. However the fry exhibited no social preference (Fig. 4a; A versus SC period, t-test, p=0.18). This result suggests that blocking NMDA receptors interferes with circuitry required for social interactions both in fry and in adult fish. However, our video-tracking revealed a significant alteration of movement dynamics in MK-801 treated fry. The treated fish produced swim bouts lacking the pre- and post-bout quiescent periods (Fig. 4b), and a near total loss of conventional forward swims (Fig. 4c). Every bout involved a change in orientation (i.e. turning), which is consistent with the "circling behaviour" observed in MK-801 treated adult fish (Swain et al., 2004), and reveals a substantial disruption to the movement pattern generator after drug treatment. These altered bout dynamics also produced an asymmetry in the pattern of social interaction (Fig. 4d); the motion of the observer fish strongly influenced the movement of the untreated SC fish (dip prior to 0 ms), but the drug-treated observer was much less influenced by the movement of the SC fish (smaller dip after 0 ms; Fig. 4d). Furthermore, the synchronicity peak at 0 milliseconds lag was abolished.

Acute exposure to high concentrations of ethanol are also known to influence the social behaviour of adult zebrafish (Gerlai et al., 2000). Consequently, we exposed three-week old fish to low (0.125%) and high (0.5%) levels of ethanol 1 hour prior to and during testing in the social assay. The influence of ethanol exposure was concentration dependent. Fish exposed to low ethanol retained a strong SPI (Fig. 4e, A vs SC period t-test, $4.0*10^{-7}$) and their bout dynamics (Fig. 4f) and composition (swims vs turns) (Fig. 4g) were unaffected. Furthermore, the strength of their BTA interaction was similar to age-equivalent untreated fish. In contrast, upon exposure to a higher concentration of ethanol, social preference was greatly reduced and the SPI distribution was not significantly different from the acclimation period (Fig. 4i, A vs SC t-test, $p=0.08$).

Remarkably, despite this loss of social preference by zebrafish exposed to high ethanol concentrations, movement dynamics (Fig. 4j), distributions of swim turns (Fig. 4k), and the strength of coordination of movements with other fish (Fig. 4l) were not substantially affected. These intriguing results suggest that social preference and interactions with other individuals, each a fundamental component of social behaviour, can be decoupled by pharmacological, and likely other, manipulations.

Discussion

We have shown that during development, zebrafish gradually develop a preference for observing age-matched conspecifics over empty chambers or younger fish. When one week old, zebrafish show little tendency to remain in a chamber with visual access to conspecifics, but by three weeks, a strong preference emerges. This preference is visually driven and does not solely depend on the size of the conspecifics, as one week old larvae do not show a preference to observe larger three-week old fish. These results suggest that social preference arises through the development of neural systems that mature, during the second and third weeks of life.

Zebrafish are still transparent during the relevant developmental stages and are thus amenable to the entire range of optical techniques for anatomical and functional investigation of whole-brain neural circuitry (Ahrens et al., 2013). Importantly, our assay has also demonstrated that visual stimulation is sufficient to drive social behaviour at three weeks of age. The presentation of visual "social" stimuli to restrained fish is much more straightforward than attempting to recapitulate the complex tactile and olfactory stimuli that are also involved in schooling/shoaling interactions (Cornelia H et al. 2012). Therefore, we are now in a position to characterize, in detail, the changes in neural circuitry that correlate with this fundamental behavioural transition. For instance, it is known that some brain areas, such as the pallium (Dirian et al., 2014), still undergo developmental changes during the time we report the social preference.

Not all fish develop a positive response to conspecifics; some individuals exhibit avoidance behaviour when other fish come into view. This intriguing result warrants further investigation and for instance, our assay could be used to determine whether fish exhibiting aversive behaviour retain this negative social bias after multiple presentations of the SC and whether different environmental, pharmacological and genetic manipulations can influence the predisposition of developing zebrafish to express more aversive or attractive social behaviour.

We found a visually mediated movement interaction between fish, present already in one week old larvae, that strengthens over subsequent weeks. The coupling of the motion of one fish to that of another is an important prerequisite for the coordinated behaviour that is prominent in groups of schooling fish (Miller and Gerlai, 2012). We have not dissected the components of the movement of one individual that trigger coupled movements in the other and, for instance, the extent to which the coupling depends upon "recognition" of the moving object as a conspecific in addition to its movement patterns. Whatever the nature of the triggers, the temporal profile of this interaction, notably its remarkable synchronicity, is reminiscent of the coordinated movements apparent in other social organisms, including humans (Sebanz et al., 2006; Richardson et al., 2007). Interestingly, the synchronized interaction we observe in zebrafish is reminiscent of other dynamic interactions. In any physical system, if two periodic movement generators are sensitive to the motion of one another, then they will act as (weakly) coupled oscillators that have a natural tendency to synchronize (Supp. Movie 5). Whether such coupling dynamics are important for the shoaling/schooling interactions of adult zebrafish, or any other species demonstrating coordinated synchronous movements, warrants further investigation. Intriguingly, disruption of low-level coordination behaviours, such as the loss of synchronized eye-blinking in autistic subjects (Sears et al., 1994; Senju et al., 2007), is now being identified as an important marker of disease that appears prior to more conspicuous symptoms, thus facilitating early diagnosis and intervention.

Whereas NMDA receptor blockade disrupted both SPI and coupled movements, alcohol disrupted social preference but left intact the ability of fish to couple their movements. This suggests that these two aspects of the overall social behavioural pattern can at least partially be uncoupled. Our ability to resolve the development of two distinct forms of social behaviour, preference and interaction, in a vertebrate model system, has tremendous potential to better characterize the influence of pharmacological and genetic manipulations. We were able to distinguish effects on social preference and interaction in zebrafish pharmacologically treated with an NMDAR antagonist (MK-801) or exposed to different concentrations of ethanol, both of which were previously shown to disrupt social behaviour in adult zebrafish (Green et al., 2012). In addition to confirming that MK-801 treatment disrupts social preference, we also found changes in underlying movement dynamics, namely a reduction in bout periodicity and the absence of conventional forward swims. This perturbation of basic movement patterns can explain the asymmetry in coordinated behaviour between MK-801 treated and untreated individuals. In contrast, fish exposed to high concentrations of ethanol exhibited no disruption of intrinsic movement dynamics and show wild-type levels of social interaction, but social preference was severely disrupted.

These results highlight our assay's sensitivity to distinct components of social behaviour, preference and interaction, components that could be separately impaired by different pathologies. Therefore, this assay is well suited for the analysis of a range of genetic(Pietri et al., 2013) and pharmacological(Poole and Hobert, 2006; Scerbina et al., 2012) manipulations that have been linked to developmental disorders affecting social behaviour.

Figure legends

Figure 1. Social preference is robust in three-week old zebrafish
a, Schematic of the behavioural setup (top). Infrared light homogeneously illuminates the behavioural arenas. Schematic of a single choice chamber (bottom left) with an observer (test) fish and multiple conspecifics as the social cue (SC). The blue lines are clear glass windows. Single frame from high-speed video recording of an experiment with three-week old fish (bottom right).
b, Examples of tracking of a one-week old (top) and three-week old (bottom) fish, in the absence (left) and presence (right) of the SC. The blue and red portions of the movement tracks are used to calculate the social preference index (SPI, indicated below).
c, Schematic depicting body orientation of the observer test fish relative to the SC chamber (top left - inset). Polar histograms, averaged across all tested fish, of body orientations of the observer fish when within the SC side of the chamber. From one to three weeks a preference emerges for the observer fish to view the SC with either the left (-45°) or right (+45°) eye. Thin lines indicate two standard errors from the mean (SEM) (one week: n=143, two weeks: n=151, three weeks: n=181).
d, Histograms of all SPIs during acclimation (left column) and SC (right column) periods across different developmental stages (one week (6-8 dpf); two weeks (13-15 dpf), three weeks (20-22 dpf). A range of positive and negative preferences are observed and red bars (SPI > 0.5) highlight strong preference for the social side, while blue bars (SPI < -0.5) highlight strong aversion for the SC (zero is marked with a dashed vertical line).
e, Histogram of all SPIs when a single conspecific served as the SC across different developmental stages.

Figure 2. Social preference requires visual observation of similarly aged fish
a, Schematic of the experiment to assess whether visual information is required for fish to show social preference. Following the acclimation period, three-week old zebrafish are presented with a SC and monitored under both normal illumination and darkness, where the order of exposure to each condition was randomized. SPIs resulting from such experiments are indicated below schematics.
b, Polar histograms of body orientations of the observer when on the SC side of the chamber during both light and dark sessions. The preference for the observer fish to orient at 45 degrees to the SC chamber is not present in darkness (thin lines indicate 2*SEM, n = 90), supporting the inference that such orientation in the light represents monocular viewing of conspecifics.
c, Histograms of SPIs for all individuals during the dark and light conditions in the presence of a single fish as SC.
d, Histograms of the SPIs of one-week old fish observing three-week old fish (left), and the SPIs of three-week old fish observing one week old fish (right). See Supp. Figure 2 for the SPIs in the absence of SC.

Figure 3. Development of the dynamics of social interaction
a, Example of the motion bout detection and alignment analysis: the left schematic shows the test chamber indicating in red the visual side of the SC fish and in blue the non-visual side of the SC fish. The plots on the right indicate how movement bouts

were analysed. Top plot shows movements bouts of the SC fish. Peaks in movement trajectories were identified with a dual-threshold algorithm (upper threshold dotted line is 3*standard deviation (3*SD) and lower threshold dotted line is 2*SD). The middle plot shows the movement bouts of the observer, test fish. The movement peaks of the SC fish were used to extract short time windows of the movement trajectories of the observer fish trajectory (2 seconds either side of the SC fish movement peak). The bottom plot shows the 'bout-triggered-average' (BTA) movement for the observer fish which was computed by averaging movements across all of the four second time windows aligned to the SC peak movement. BTAs were computed separately depending on whether the observer fish could view the SC or not (left schematic).

b, The average bout motion time-course for all SC fish, normalized to the peak movement of each fish , at different developmental stages. The average bouts are overlaid to highlight changes in the kinetics between one and three weeks of age.

c, Scatter plot presentation of all bouts, where each bout is represented by single point based on the position and orientation change that occurred for that bout (n=164 fish).

d, BTAs of one- to three-week old observer fish motion aligned to movement bouts of single SC fish (red) or plotted when the SC was not visible (blue) (one-week: n = 106, two-week: n = 136, three-week: n = 163).

e, BTAs for fish monitored in darkness when on the same or opposite side of the SC (n = 90).

Figure 4. Exposure to NMDA receptor antagonist or ethanol disrupts social preference and differentially impairs social interactions

a-d) analysis of fry treated with 100 μM MK-801 NMDA receptor antagonist.

a, Histogram of SPIs revealing no apparent preference for the SC and (inset) body orientations showed little or no direction towards the SC chamber (zero position). SPIs during the acclimation periods are shown in Supp. Figure 2d.

b, Average motion bout profile for MK-801 treated fish. Relative to untreated controls (grey plot), there is a reduction in the pre- and post-bout quiescent periods and consequently the periodicity of bout generation.

c, Scatter plot presentation of all bouts from all tested fish, where each bout is represented by single point based on the position and body orientation change that occurred for that bout. MK-801 treatment results in a conspicuous reduction in forward swimming bouts ('0' position on X-axis).

d, Bout-triggered averages (BTA) of MK-801-treated observer fish when the SC fish is visible (red plot) or not visible (blue plot). There is a disruption of normal movement interactions (compare to comparable plots in figure 3 or in h and l below) and the abolishment of behavioural synchrony at 0 seconds offset.

e-h) Comparable analyses as in a-d of fry treated with 0.125% alcohol

e, Plot of SPIs showing that social preference (red) remains and (inset) orientations were directed towards the SC chamber.

f-h, Average motion bout profiles (f), body orientations (g) and BTA plots are all similar to untreated three-week old zebrafish.

i-l) Comparable analyses as in a-d of fry treated with 0.5% alcohol

i, Analysis of SPIs showing social preference is severely disrupted and (inset) body orientations are less strongly directed towards the SC chamber.

j-l, Average motion bout profiles (j), body orientations (k) and BTA plots (l) are all similar to untreated three-week old zebrafish.

Materials and Methods

Zebrafish husbandry. AB strain zebrafish (*Danio rerio*) to be tested were bred, raised and housed in the same environment. All fish were obtained from natural spawning and housed in groups of roughly 50 fish, and kept at 14h light/10h dark cycle. Fish were fed two times per day from 4 dpf with dry food diet from SAFE Diets (particle size 50-100) and twice with salt water rotifer (Brachionus Plicatilis) until 10 dpf; then twice a day with dry food diet (particle size 100-200), and with a combination of salt water rotifer and brine shrimp (Artemia salina) until 15 days; finally twice a day with dry food diet (particle size 200-300) and with brine shrimp until used in the experiments. All the fish run in the behavioural assay were fed in the morning. The experiments described were approved by local ethical committee and the UK Home Office.

Behavioural assay. Experiments were performed in a custom-built behavioural setup (Fig. 1a) that was assembled from structural framing (Misumi, DE) and optomechanics (Thorlabs, USA). The videography system comprised a high-speed camera (Flea3, PointGrey, CA), infrared LED backlight (Advanced Illumination, USA), infrared filter (R70, Hoya, JP), and a vari-focal lens (Fujinon, JP). Fish were imaged in a custom-built behavioural arena that was fabricated with a laser-cutter from 5 mm thick opaque white acrylic, sealed with silicone, and with glass window partitions; the multi-chamber design is shown in Fig. 1a. The arena was supported on a transparent base covered on one side with diffusive gel paper (Rosco Cinegel, USA). It was illuminated with visible light by homogenously projecting a white rectangle, via a 45° infrared cold mirror positioned between the chamber and IR illuminator, onto the base of the assay using a laser light projector (Microvision, ShowwX+, USA). For all experiments, the entire behavioural apparatus was enclosed in a light-tight enclosure, and for the dark experiments, the visible background illumination was removed.

Acquisition software: Fish in six individual chambers were contemporaneously tracked in real-time using custom written workflows in Bonsai, an open-source C# data stream processing framework (Goncalo et al., 2015 published in Front. Neuroinform). For each chamber, the image was cropped, background subtracted, thresholded, and the centroid found, characterized (position, orientation, and heading), and stored in a CSV file. The video was also saved with H.264 compression for subsequent offline analysis.

Data analysis: Social Preference Index (SPI) was calculated by subtracting the number of frames in which the fish was located within the region near the conspecific SC (area highlighted by the red tracking in Fig. 1) from the number of frames spent in the equivalent region on the opposite side of the chamber (blue tracking in Fig. 1). This difference was then divided by the total number of frames recorded in the two analysis compartments during the experiment, resulting in a value varying between -1 and 1. The SPI during the acclimation period, for which there is no SC, was computed

with reference to the randomized side of the chamber on which the SC would be added in the subsequent experimental phase.

The compressed video from each experiment could be repeatedly re-analysed using custom written bulk-processing routines in Python. A motion trajectory for each fish was computed by first segmenting the binary particle for each fish and then measuring the change in pixel values for that particle from one frame to the next. This resultant frame-by-frame segmented motion trajectory provided a very stable signal for identifying the peak of individual bouts and then testing for interaction between the observer and SC fish.

Statistical analysis:
Two-tailed Student's t-tests were used to compare the group distributions for different test conditions. The distributions for the most subtle A vs SC comparisons (1 week old, MK, EtOH, etc.) are Gaussian. We therefore use the same statistic throughout, even in the case of the three week old fish that are clearly social and whose distribution (during the SC) reaches the SPI bound (+/- 1).

Drug treatments: MK-801: 100 mM stock solution was prepared by dissolving MK-801 (M107; Sigma-Aldrich) in 100% DMSO (D2650; Sigma-Aldrich) and stored at -20°C. The drug was administered for 1 h prior the experiments by diluting the stock solution in fish water in order to obtain a working concentration of 1μM. Zebrafish were washed with fish water before placing them in the chamber for recordings.
For ethanol experiments, low (0,125%) or a high ethanol (0,5%) concentrations were obtained by diluting ethanol in fish water. Fish were exposed with one of the two ethanol concentrations for 1 h prior to, and during experiments.

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Author contributions: ED, ARK and SWW designed the project; ED performed experiments; GL created the software to acquire and analyze the data; ED and ARK analyzed data; ED, ARK and SWW wrote the paper.

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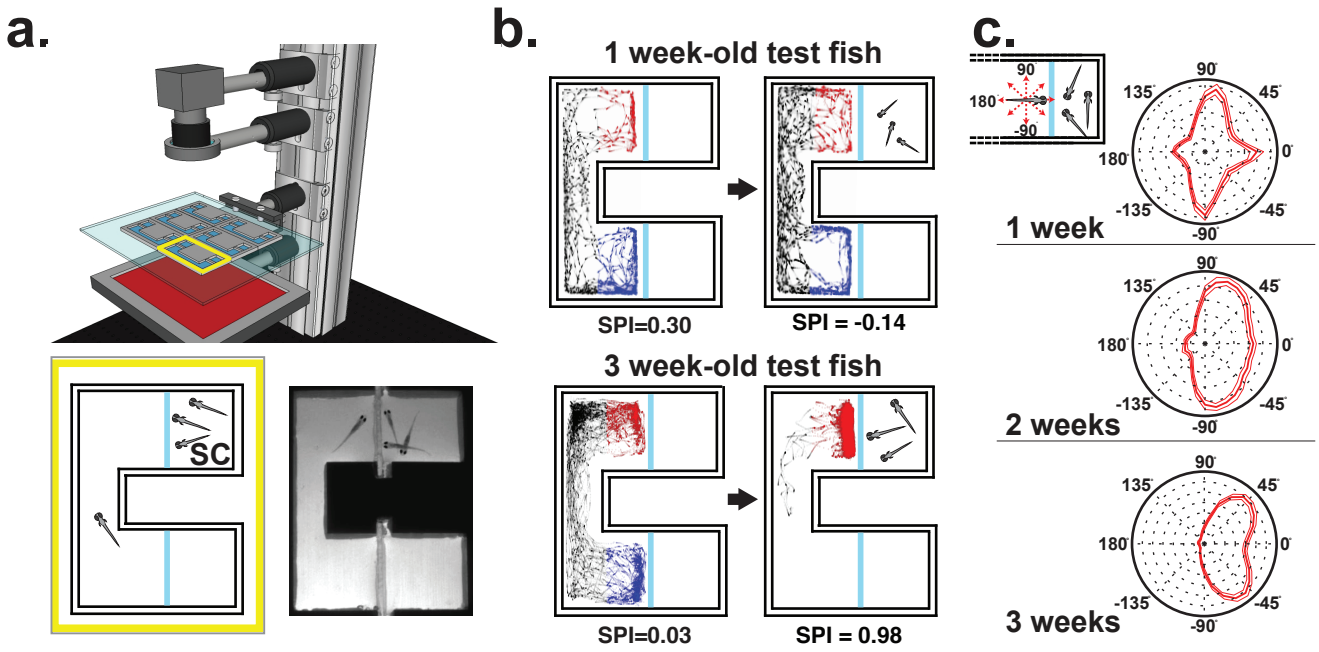
Figure 1



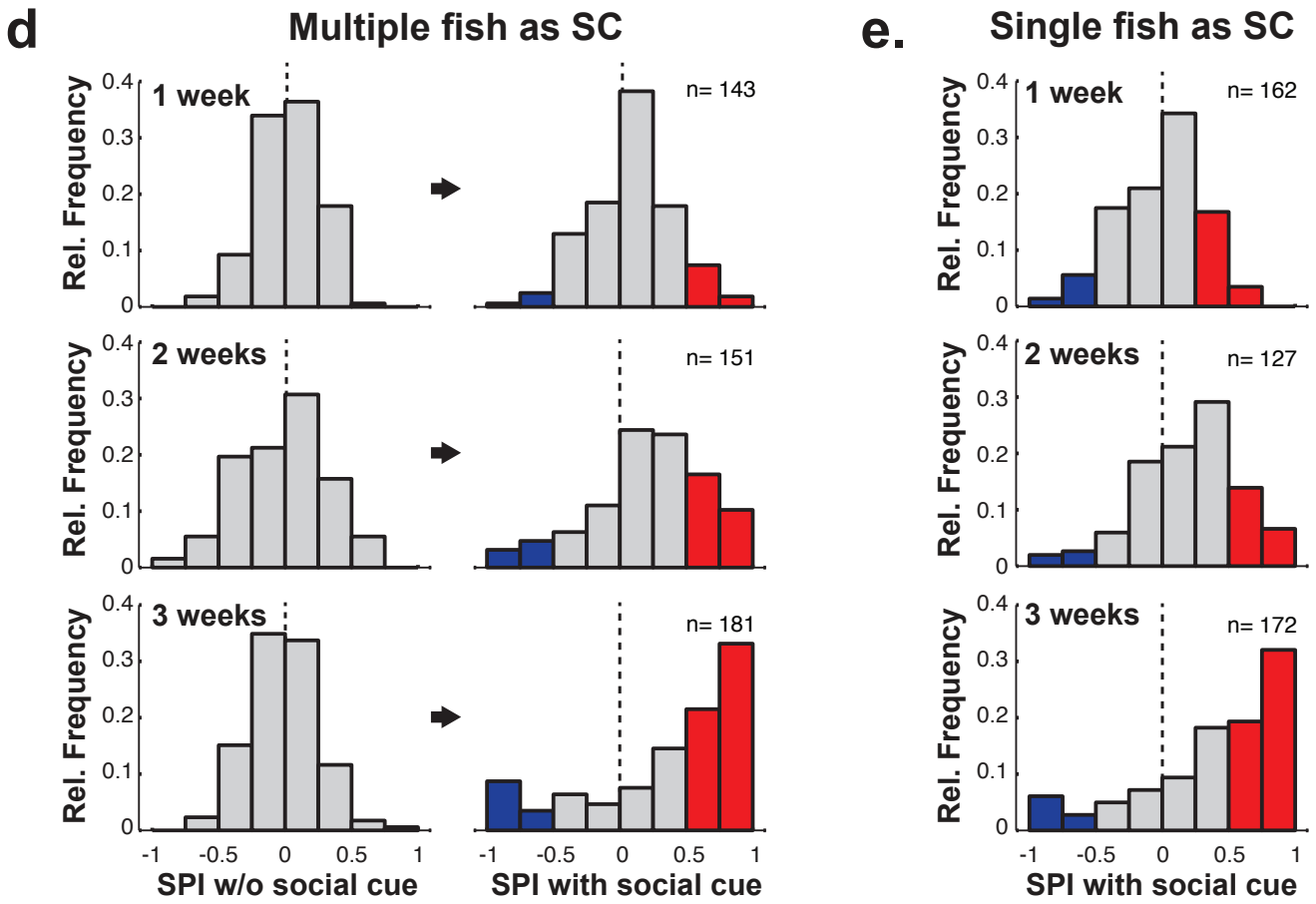


Figure 2

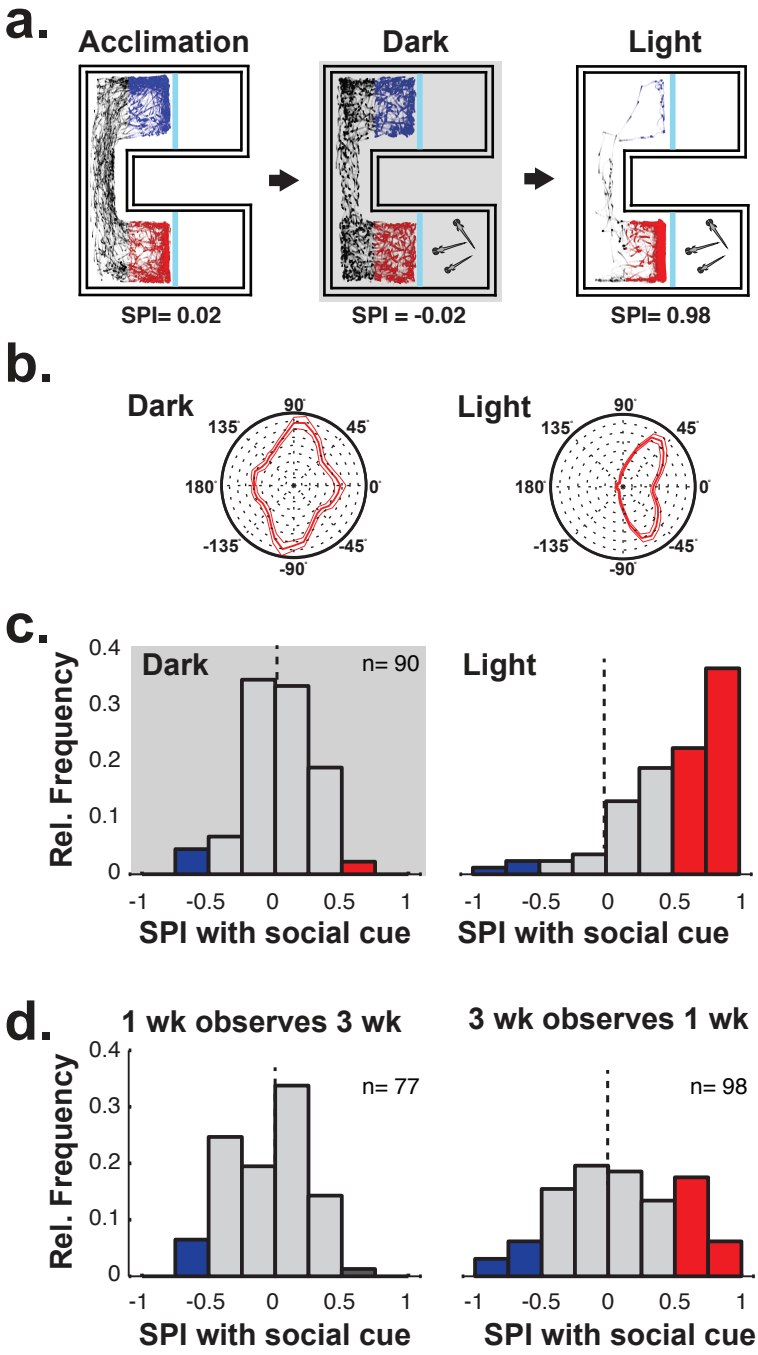


Figure 3

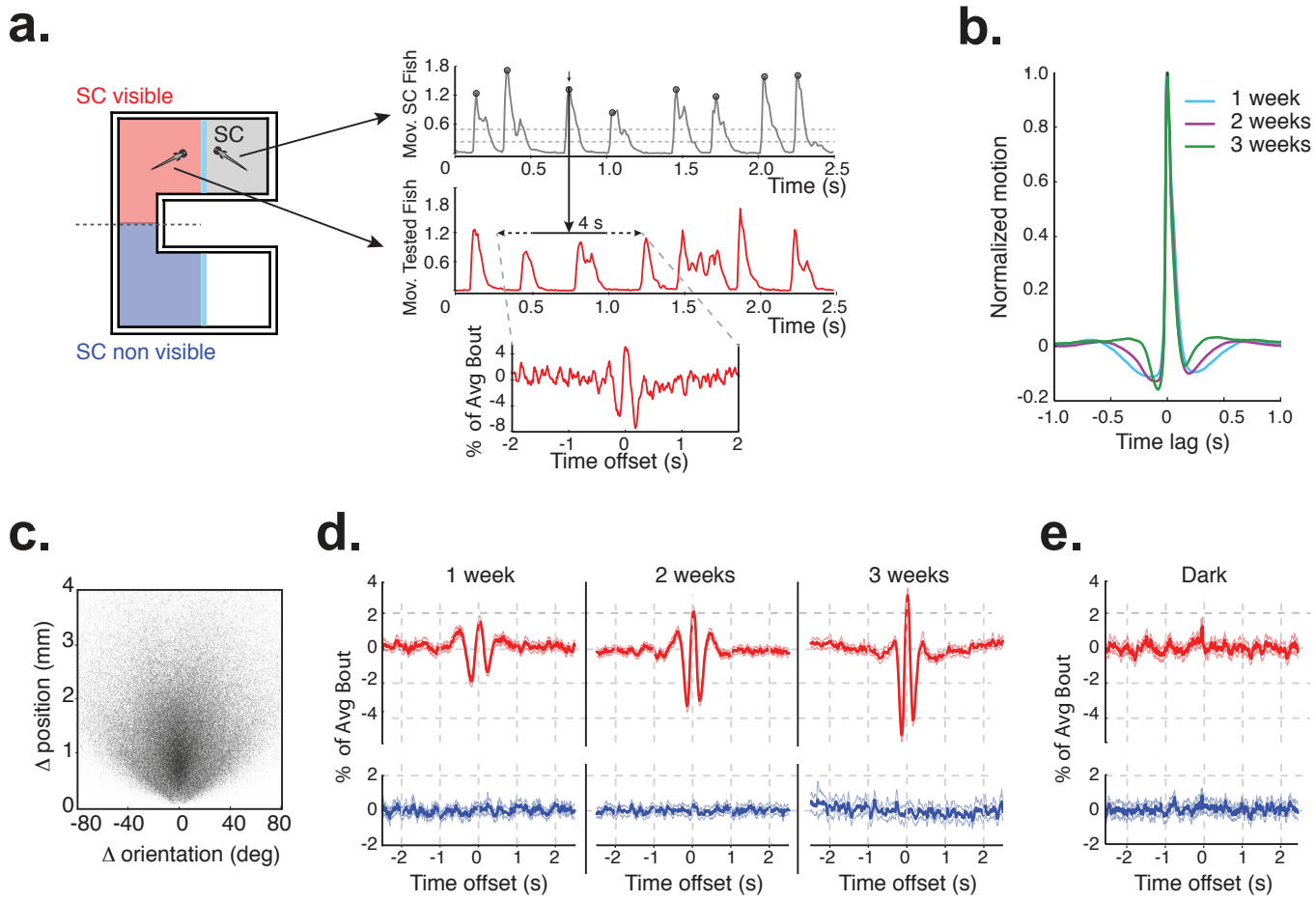


Figure 4