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Discrimination reversal and attentional sets in zebrafish (*Danio rerio*)

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Abstract

The potential of zebrafish as a comparative model in behavioural neuroscience is currently hampered only by the lack of reliable and validated behavioural assays available to researchers. In the present experiment, we describe the performance of zebrafish in a test of attentional set formation. The fish were initially trained on a two-choice colour discrimination. Upon reaching acquisition criterion, the reinforced alternative was switched to the previously unreinforced alternative. Again, upon reaching criterion, the cues were replaced with a novel pair of colours (intra-dimensional shift) and reversed again on reaching criteria. We found that zebrafish show a steady decrease in trials-to-criteria over the four phases of the experiment, suggesting that they are forming and maintaining an attentional set, as has previously been demonstrated with mammals. Reversal learning deficits have been implicated in a variety of human psychological disorders (e.g., disorders of impulse control) and as such, we propose that performance of zebrafish in this procedure may represent a useful comparative model to complement existing rodent models.

Keywords

Zebrafish; Reversal learning; Attentional set; Attention; Intra-dimensional shift

1. Introduction

Zebrafish (*Danio rerio*) are becoming an increasingly popular model system in behavioural neuroscience [1,2]. The progress of the species in this respect is limited only by the rate at which laboratories develop and publish detailed behavioural protocols, such that any phyletic differences in learning capabilities or performance characteristics (qualitative or quantitative) can be scrutinised [3–9]. While quantitative differences in learning between vertebrate species are to be expected, qualitative differences in learning could severely compromise the translational relevance of animal models [10]. One such potential qualitative difference has been suggested in terms of the performance of fish in reversal

learning procedures [11–13]. Different species may solve reversal learning problems in different ways, which may indicate different degrees of behavioural flexibility, the ability to ‘learn rules’ [10] or, more specifically, a shift in stimulus control from simple stimulus-reinforcer learning to a more generalised, rule-based approach [14].

The typical procedure for examining reversal learning involves, first, an animal being trained on a simple discrimination between two stimuli, $A+ B-$. When the animal reaches a set criterion (e.g., 80% correct in a given number of choices, or n consecutive correct choices) the reinforced alternative is switched with the previously unreinforced alternative (i.e., $A- B+$). In many species (e.g., rats, pigeons, monkeys and humans), despite a high number of errors typically during the initial reversal, the number of errors decreases as a function of subsequent reversals [12,15,16]. A number of potential reasons for this systematic improvement in performance have been suggested, but appear to be related to executive processes during continued learning [17].

Although initial attempts to characterise progressive improvements in reversal learning in fish indicated that there may be qualitative differences between fish and other vertebrates [e.g., pigeons, rats; 12], others demonstrated that, given the right conditions (e.g., the inclusion of a centre-key or exposure of the fish to the incorrect stimulus after the choice was made), fish are capable of improvement over multiple reversals [18–20]. The purpose of the current paper is primarily to describe how zebrafish perform on reversal learning and intra-dimensional (ID) set-shifting and indeed whether they are able to form, maintain and then shift an attentional set [14]. Although others have found that zebrafish are capable of learning a colour discrimination and of discrimination reversal [21], no one has yet examined if this species shows any evidence of the acquisition of a ‘learning set’. In addition, we included a test of ID set shifting to see if the fish generalised the rule to new stimuli. In the light of a previous demonstration that gold-fish show progressive improvement in reversal learning when the incorrect stimulus remains active during time-out [19] we designed the present discrete-trial experiment such that the fish remained confined in the presence of the incorrect stimulus for 10-s following its choice.

2. Methods

2.1. Subjects

Fifteen adult (~1-year-old) zebrafish from the common AB wild-type strain were used for the experiment. All of the fish had previously experienced training on two-choice visual discriminations (red vs green; one week of training), although none of the fish had experienced the particular colour-pairs used in the present experiment. All the fish had been bred and reared in the aquarium facility at Queen Mary University of London, licensed by the UK Home Office. During the course of the experiment, the fish were housed in pairs, with a room temperature of ~28 °C, and a 14 h:10 h light:dark cycle. Testing was conducted in the same room to avoid stress during transfer. Fish were fed only during behavioural testing, during which they received brine shrimp. The amount of brine shrimp received during training typically equated to more food than the fish would be fed. A session comprised 30 discrete trials, so that the maximum number of shrimp was 300 μ l (see below) and typically a fish earned 100–150 μ l. If a fish made more than 10 errors during a session,

their feed was supplemented after testing in order to ensure feeding was consistent between fish. After they had completed the behavioural testing, the fish were returned to the facility breeding stock.

2.2. Apparatus

Fig. 1 depicts the apparatus used to train the fish. During training trials, the fish was first confined to the holding area, after which the barrier was lifted and the fish was allowed to explore the tank. The same barrier also acted as the barrier to restrict the fish in the food-delivery area. Reinforcers (brine shrimp suspended in aquarium-treated water) were delivered via a syringe with catheter-tubing attached to the needle ($d = 1$ mm), mounted on a wooded block with a steel bolt positioned behind the plunger (Fig. 2). This facilitated small amounts of food (~ 10 μ l) being delivered precisely by turning the screw a quarter turn. Food delivery tubes were located in both of the food delivery areas to avoid the fish learning the location of the tube as CS+. The colour exemplars used are outlined in Table 1. The exemplars were tested in a spectrometer to assess their peak absorbency, and we chose colours that were found to have similar luminance to control for salience (data not shown but available on request). The colour exemplars were printed on card and laminated. All procedures were carried out by hand by two experimenters.

2.3. Design and procedure

2.3.1. Phase 1 – pretraining and habituation—Fish were acclimated to the holding room for 1-week prior to the start of training. In addition, the fish were habituated to the training tank for two half-hour sessions prior to their first training session. During this time, food was made available if the fish entered the food delivery areas. The fish was not confined to this area at any time during habituation sessions. Following habituation, the fish were trained to enter the food delivery areas. The fish was restricted to the holding area of the tank, after which the barrier was lifted. Entry to either of the food delivery areas was reinforced. During reinforcement, the fish was restricted to the food delivery area. Following consumption of the food (typically ~ 10 -s) the fish was released from the food delivery area and stewarded back to the holding area with the barrier. All sessions commenced with the fish being captured in a net in its home tank and being placed into the test tank. It was then left to acclimate for 5 min prior to the trials commencing. During this time, the fish was not confined to any area of the tank.

2.3.2. Phase 2 – initial discrimination—Each session comprised 30 discrete choice-trials. The fish was placed in the experimental tank (no exemplars) and allowed to swim around unrestricted for 30 s. After this initial 30 s, the session began when the fish swam into the holding area of the tank. The fish was confined to the holding area (closest to the experimenter) for 30 s. The barriers were then lifted and the fish was free to explore the choice area. Once the fish entered the food delivery area and approached the colour exemplar, the barrier was lowered and the fish restricted to the food delivery area for 10 s after its choice, after which time the barrier was lifted. If the fish had made the correct selection, food was delivered. If the choice was incorrect, the fish was still restricted to the food delivery area for the same time period (10-s). When the fish left the food delivery areas and entered the holding area, it was then confined for the period of the inter-trial interval

(10-s). Learning criterion was set at the fish achieving six consecutive correct trials. The criterion had to be reached within one 30-trial session (i.e., correct responses were not carried over to subsequent sessions).

2.3.3. Phase 3 – reversal 1—Once the fish reached criteria for learning for the first colour, the rule was reversed such that the unreinforced alternative (negative conditioned stimulus [CS–]) became the reinforced alternative (CS+). Training then continued as before, until criteria for learning was reached (6 consecutive correct choices).

2.3.4. Phase 4 – ID shift—Following the fish successfully reaching criterion for the first reversal, an ID shift was performed (see Table 1 for counterbalance information). Here, the fish were presented with a novel pair of exemplars and trained as before. Criteria for learning remained the same at six consecutive correct trials. Once the fish learned the rule for the new set of targets, reversal of the targets took place.

2.3.5. Phase 5 – reversal 2—Upon reaching criterion for the ID shift, the rule was again reversed, with CS+ becoming CS–. Again, learning was operationalised as six consecutive correct trials.

2.4. Analysis

Statistical analyses were carried out in PASW (Version 18). Trials-to-criterion and between-phase comparisons of approach latency and error-rate data were subjected to one-way within-subjects analyses of variance (ANOVAs), each with 4 levels (initial discrimination, first reversal, ID shift, second reversal). Between-day approach latency and error-rate data were unsuitable for ANOVA owing to each subject completing the four phases at different rates, and as such we used a linear mixed model (LMM) which is able to account for missing repeated-measures data [22]. In the LMM, F values were estimated by restricted maximum likelihood (REML). Here, the within-subjects factor was Day (18-levels: Day 1–Day 18). In all cases, post hoc analyses were corrected for multiple testing (Bonferroni method). Statistical tests are reported with respect to an α level of 0.05. Descriptive statistics are reported as mean ($\pm$ SEM) unless otherwise indicated.

3. Results

Fig. 3 displays the number of trials to criterion (six correct trials in a row) for fish during the four stages of the experiment. As is clear, there was a reduction in the mean number of trials as the stages progressed, with fish requiring nearly seven-times as many trials in the first stage as in the last (the second reversal phase), and the first reversal and ID shift were completed in successively fewer trials. This was confirmed with a one-way within-subjects ANOVA ($F_{3,42} = 24.68$). Bonferroni-corrected post hoc comparisons revealed that there were significant differences between the initial discrimination and the first reversal ($p < 0.05$), the ID shift ($p < 0.001$) and the second reversal ($p < 0.001$). There was no difference between the first reversal and the ID shift ($p = 0.80$), but there was a difference between the first reversal and the second reversal ($p < 0.01$) and between the ID shift and the second reversal ($p < 0.05$).

Fig. 4a displays the mean approach latency for correct trials in the different phases of the experiment and Fig. 4b displays the approach latencies on a day-by-day basis (i.e., independent of phase). In terms of the comparison of the phases (Fig. 4a), approach latency appears to reduce after the initial discrimination, but does not change between the first reversal and the subsequent sessions. The differences were confirmed with a one-way repeated measures ANOVA ($F_{3,42} = 5.76$). Post hoc comparisons adjusted for multiple tests revealed that there were significant differences between the initial discrimination and the first ($p = 0.05$) and second ($p < 0.02$) reversals. There were no significant differences between the other conditions ($ps > 0.2$). With respect to the day-by-day comparison (Fig. 3b), it is clear that the latency to approach reduces fairly rapidly over the first four test days, and levels thereafter. This was confirmed with an LMM ($F_{17,170} = 5.63$). Post hoc comparisons revealed that there was a significant decrease in latency between day 1 and days 3–13 ($ps < 0.03$), but this effect disappeared in days 14–18 ($ps > 0.3$).

Fig. 5a depicts the accuracy of responding between the different phases of the experiment, and Fig. 5b the accuracy day-to-day. With respect to changes in accuracy rate between phases, there appears to be little change across the phases. A within-subjects ANOVA was performed and the sphericity assumptions could not be met; as such, the degrees of freedom in the model were estimated by the Greenhouse–Geisser adjustment. The ANOVA, revealed a marginally non-significant effect of phase ($F_{2,28} = 2.96$, $p = 0.07$). With respect to the day-to-day changes in accuracy, there appears to be no change throughout the experiment. This was confirmed with a LMM ($F < 1$).

Finally, we carried out an analysis of the relationship between accuracy rate and latency to approach within each phase of the experiment. Overall, there was a negative correlation between latency and accuracy rate ($r = -0.17$). However, when this was examined on a phase-by-phase basis, there was a medium-strong correlation between latency and accuracy rate during the initial discrimination ($r = -0.32$; Fig. 6), but not in the other phases ($rs < 0.1$).

4. Discussion

The results of the current study support the assumption that zebrafish are capable of forming, and maintaining, an attentional set. In addition, it suggests that zebrafish do show progressive improvement over time under these conditions, with the number of trials to criterion reducing as they progressed through the stages of the experiment. This replicates previous research showing that zebrafish are capable of (a) distinguishing between two colours when reinforced with food (b) reinforcement of previously unreinforced alternative results in reversal of the discrimination and (c) zebrafish improve their acquisition performance over time [21]. Our findings extend the Colwill et al. [21] study, however, in that in the present study all our fish took part in all the learning phases (i.e., initial colour discrimination, reversal, ID shift, reversal) whereas Colwill et al. [21] tested only one discrimination reversal per group of fish. As such, we have shown evidence that given the appropriate training and testing conditions, zebrafish are able to break from simple S–R stimulus control and acquire a ‘rule’, as demonstrated in other species [19,20].

It could be argued that the decrease in trials to acquisition over the four phases of the experiment were not the result of rule learning, but rather were representative of habituation to the test environment and/or handling. However, this seems unlikely as we found that the fish showed a significant decrease in approach latency over the course of the initial discrimination, but further decrease was not evident thereafter. This suggests that any habituation happened during the initial discrimination. In addition, our results demonstrated that although the fish showed a decrease in approach latency over the course of the 18-day experiment, there was no significant increase in accuracy rates. There was, however, a negative correlation between accuracy rates and approach latency, which was strongest in the baseline condition. It would be unwise, based solely on these correlational data, to make inferences from this about fish learning in general. Nevertheless, taken together, this suggests latency to approach may be used as a dependent variable in this procedure, but at present there is not enough evidence to use accuracy rate in this context.

Lesion studies in rats have demonstrated that reversal learning is mediated by the orbitofrontal cortex [OFC; 23] and extra-dimensional set shifting by the medial prefrontal cortex [mPFC; 24,25]. Impairments in reversal learning are related to deficits in impulse control and this relationship is mediated by the OFC [17,26,27]. More specifically, 5-HT depletion from the OFC impairs reversal learning and results in more perseverative errors during reversals [28], suggesting that OFC 5-HT may mediate behavioural flexibility. There have been various attempts to find anatomical and functional homology between the brains of zebrafish and mammals [7,29]. For example, using tyrosine hydroxylase (TH) immunocytochemistry, Rink and Wullimann [29] described the ascending dopaminergic pathways in the zebrafish brain, speculating homologies for the ventral tegmental area (posterior tuberculum), the striatum (ventralis telencephali) and prefrontal cortex (pallium or dorsomedial telencephalon). Ascertaining the functional relevance of these homological and anatomical similarities, however, relies heavily on development of valid and reliable behavioural assays in the zebrafish, combined with lesion studies and/or use of the extensively available mutant lines [30].

There have been some attempts to characterise the neuropsychology of reversal learning in fish. Gross telencephalic ablation in paradise fish (*Macropodus opercularis*) caused a significant deterioration in reversal learning, but caused no associated sensory or motor deficits [31]. In addition, Farr and Savage [32] ablated the telencephalon of goldfish (*Carassius auratus*) and tested various aspects of Pavlovian and instrumental learning. Interestingly, the lesions produced very little deficit in acquisition of a variety of procedures, however, there were differences in avoidance learning and in second-order conditioning. This suggests that the telencephalon may be functionally related to the mammalian limbic system, but also may play a role in cognitive modularity in the fish [33]. Again, until more sophisticated behavioural assays are conceptualised and reported, it will be difficult to be sure of the full extent of the capabilities of zebrafish as comparative models in behavioural neurobiology.

It is important to note that although care was taken to ensure that procedures were carried out in a controlled and standardised manner, the fact that we did not use automation leaves open the possibility of experimenter effects. It is crucial that we move towards automated

procedures to reduce these potential confounds, but also to increase the throughput of behavioural testing of fish.

5. Conclusions

Reversal learning deficits, and general deficits in attentional set formation, are key symptoms displayed by humans suffering a variety of psychological disorders related to impulse control [17,34]. The results of the current study suggest that there may be a role for zebrafish in the future as a useful comparative model, to complement the existing rodent models, to study the aetiology, development and pathogenesis of some of these disorders.

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Fig. 1. Schematic of the testing tank used for the two-choice discriminations.

The subject was first restricted to the holding compartment, and allowed access to the rest of the tank at the start of a discrete trial. A choice was made when the fish swam into one of the compartments and approached the stimulus therein.

Fig. 2. Food delivery system.

The food used to reinforce the fish was artemia suspended in aquarium water. It was delivered by hand by the experimenter if the response was correct.

Fig. 3. Trials to criterion during each of the four phases of the experiment. Shared letters denotes $p > 0.05$.

Fig. 4. (a) Latency to approach the stimulus areas during each of the four phases of the experiment. $*p = 0.05$; $p < 0.02$. (b) Latency to approach the stimulus areas during each training day of the experiment (i.e., regardless of phase).**

Fig. 5. (a) Accuracy (proportion of correct responses) during each of the four phases of the experiment. (b) Accuracy (proportion of correct responses) made during each day of training, regardless of the phase of the experiment.

Fig. 6. Correlation between accuracy (proportion of correct responses) and latency to approach the exemplars (s) during the initial discrimination phase.

Table 1
Colour pairs used as exemplars during training according to counterbalance condition.

Condition	Initial Discrimination	Reversal 1	ID shift	Reversal 2
A (Ss 1,2,3,4,5)	Light yellow (CS+) Light green (CS-)	Light yellow (CS-) Light green (CS+)	Blue (CS+) Magenta (CS-)	Blue (CS-) Magenta (CS+)
B (Ss 6,7,8,9,10)	Red (CS+) Dark yellow (CS-)	Red (CS-) Dark yellow (CS+)	Blue (CS+) Magenta (CS-)	Blue (CS-) Magenta (CS+)
C (Ss 11,12,13,14,15)	Blue (CS+) Magenta (CS-)	Blue (CS-) Magenta (CS+)	Red (CS+) Dark yellow (CS-)	Red (CS-) Dark yellow (CS+)