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Shoaling promotes place over response learning but does not facilitate individual learning of that strategy in zebrafish (Danio rerio)

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Abstract
Background: Flexible spatial memory, such as “place” learning, is an important adaptation to assist successful foraging and to avoid predation and is thought to be more adaptive than response learning which requires a consistent start point. Place learning has been found in many taxonomic groups, including a number of species of fish. Surprisingly, a recent study has shown that zebrafish (Danio rerio), a common species used in cognitive research, demonstrated no significant preference for the adoption of either a place or a response strategy during a plus maze task. That being said, a growing body of research has been looking at how group living influences navigational decisions in animals. This study aims to see how zebrafish, a shoaling species, differ in their ability to perform a maze task when learning in a shoal and as an individual.

Results: Results suggest that shoals of zebrafish are able to learn to perform the spatial memory task in a significantly shorter time than individual fish and appear to show place learning when tested from a novel start point. Interestingly, zebrafish who were trained first in a shoal but were then tested as individuals, did not show the same level of consistency in their choice of navigation strategy.

Conclusions: These findings suggest that shoaling influences navigation behaviour, resulting in faster group learning and convergence on one spatial memory strategy, but does not facilitate the transfer of the strategy learned to individuals within the shoal.

Keywords: Zebrafish, Danio rerio, Spatial memory, Spatial cognition, Navigation, Shoal

Background
Being able to navigate through a familiar environment is critically important in the lives of all animals. Successful spatial memory helps an animal find food and avoid predation [1–3]. Previous studies have suggested that animals can use a variety of methods to find their way through an environment [4–6], using two predominant mechanisms for encoding spatial locations to memory, namely egocentric and allocentric [7, 8]. Allocentric encoding, so called “place learning”, is thought to be more complex and cognitively demanding as it involves building up relationships between multiple features within the environment and is often synonymous with a cognitive map [6, 8], although relationships between multiple features can arise through associative mechanisms [9–13]. On the other hand, egocentric encoding is based more on learning a particular set of responses to reach the goal, for example, learning a route and is thus often referred to as response learning [8, 14]. By creating complex mental representations of the landmarks within the environment not solely reliant on associative processes formed with single cues, allocentric spatial memory is thought to be a more flexible strategy as it allows the animal to locate a goal from a novel start point without the need to recapitulate a previously learned route [15]. Previous experiments have
Individuals within a group may prefer different strategies for facilitating spatial learning, and it is possible that group-moving animals are presented with conflicting directional preferences among group members. How the individuals, and the group as a whole, deal with this potential difference in spatial memory mechanisms within the group poses an interesting question. Although the role of group living in navigational decision-making has been relatively understudied, it has started to receive more attention in the last 10 years. The effect of group living on navigational accuracy [19] and/or collective decisions [20] has been explored theoretically. Empirical studies have looked at how information affects group cohesion [21–24] and how differential experience influences group decisions [25, 26]. However, the question of whether being a member of a group influences overall choice of navigational strategy remains unanswered. Related to this is how group membership influences the spatial learning of individuals within the group. Some evidence suggests that individual guppies can learn foraging routes from others [27], but in other cases, the so called “passenger effect” occurs (e.g. in pigeons), in which following another animal to a goal does not facilitate individual learning [28] (although see [29]). In general however, the extent to which behaviours formed as a group are retained by individuals, is poorly understood [30].

Fish are often selected as preferred animals of study as they are relatively easy to keep compared to other vertebrates (e.g. mammals) and also have comparative cognitive ability [31]. Indeed, the general use of fish in learning and memory experiments has significantly increased in recent years [32, 33]. Shoaling is also a common occurrence in fish species which makes them ideal models for studying group behaviour [31–34]. A recent study by the authors used a plus maze task to explore the individual spatial memory of four different species of fish. The results showed that three species of fish (killifish, goldfish and Siamese fighting fish) demonstrated a preference for the use of the more complex, allocentric place strategy, whereas the fourth species, zebrafish, showed no significant preference for either a place (allocentric) or a response (egocentric) strategy. Furthermore, the zebrafish were found to take significantly longer to learn the task than any of the other three species [16]. The fact that zebrafish are a naturally shoaling species [34–36] presents an opportunity to investigate how different individual preferences may influence the overall strategy of the shoal and indeed the general cohesiveness of the group.

With this in mind, our study aims to address two questions: [1] does shoaling result in a more consistent navigation strategy, and; [2] does it facilitate or impede transfer of learning to individuals within the shoal? To answer these questions, we will compare the predominant navigation strategies (allocentric (place learning) vs egocentric (response learning)) adopted by individual zebrafish, shoals of zebrafish, and individual zebrafish trained in shoals using the classic plus maze paradigm.

Methods

Subjects

Twenty individual zebrafish (Danio rerio) were trained and tested as part of a previous experiment [16] and form part of the analysis of the results presented here. Forty shoals consisting of five fish (200 individual zebrafish in total) were tested during the course of the current experiment. Two groups of 20 shoals were tested under two different experimental procedures on the probe trial (see below). The fish were all adults although exact age was unknown. The sex of the fish was not known. All animals were experimentally naïve and were commercially sourced from two local suppliers, due to availability of stock (the 20 trained and tested as individuals from Exotic Aquatics, Belfast, N. Ireland and fish in shoals from Grosvenor Tropicals, Lisburn, N. Ireland). All fish were introduced to the laboratory a minimum of 1 week before any experiments began. This was to allow the animals time to acclimatise to laboratory conditions and also to allow natural shoaling to occur in the relevant fish.

Housing conditions

All apparatus was commercially sourced (from sources listed in the Subjects section and also from Maidenhead Aquatics, Newtownabbey, N. Ireland). Individual zebrafish were each housed separately in 2 L glass jars) during the course of experimentation for identification purposes. A maximum of 10 jars were placed together on a heat mat at any one time so that individuals could see other conspecifics. Shoals were kept in 25 L tanks with a density of five individual fish in each. Water was maintained at an average temperature of 25 °C. When not completing experiments, all fish were fed commercial flaked food. pH and waste levels in all tanks were monitored regularly using API Freshwater Master Test Kit and water changes were carried out on a regular basis.
Waste levels were kept within safe ranges (0 ppm ammonia & nitrite; <40 ppm nitrate). pH range was maintained at a range of 7.7 ± 0.3. All fish were maintained in a 13:11 h light: dark cycle at all times during the laboratory.

Experimental design

Apparatus
The exact apparatus used in a previous related study [16] was also used during the course of these experiments and consisted of a plus maze made from acrylic Perspex panels glued to the inside of a square tank measuring 63 cm × 63 cm × 43 cm (Fig. 1). pH, waste levels and temperature were maintained at the same levels as the housing conditions and water changes were also carried out regularly in the experimental tank. All trials (both training and probe) were recorded using a Sony HDR-X190E Handycam video camera mounted above the tank. Trials were timed using a standard stopwatch.

Experimental design

Training-individuals
Training was conducted between 19/03/2013 and 14/03/2014. A training block consist of a total of 10 trials. Each fish would complete a maximum of one training block per day. Training began at 9 am and carried on until each fish had completed one block. Fish were randomly assigned to receive a bloodworm reward at the arm either to the left or to the right of the start arm (n = 10 for each side). A trial was considered complete when the tail fin of the fish had passed fully into either arm of the maze. If the fish swam to its assigned rewarded arm, it would receive bloodworm immediately administered by the experimenter using tweezers and the fish would then be moved back to the start arm for the next trial. If the fish turned to their correct side in 8 out of 10 of these trials, this training block was considered ‘successful’. If the fish had 3 consecutive ‘successful’ training blocks, then the fish was considered to have reached training criterion. The probability of at least 24/30 trials correct occurring by chance is <0.0001 and this is consistent with other studies using this method as a criterion e.g. [6]. If the fish swam to the unrewarded arm, the exit from that arm would be blocked using a removable piece of Perspex and the fish would receive a two-minute “time out” (no reward given), to mimic the amount of time the fish spent feeding before being moved back to the start arm for the next trial.

The water in the tank was disturbed between each trial to help minimise the risk of the fish using olfactory cues to navigate. The tank would also be fully filtered for a minimum of 20 min between each individual training block. Potential intramaze visual cues were reduced or eliminated where possible, e.g. the heater was removed from the tank during experiments, and the tubing of the external filter was mirrored in the maze layout using additional pieces of tubing. Outside the maze, there was a wall at the end of the left arm, while there was no wall at the end of the right arm. Potential extramaze cues included housing tanks and pieces of paper and plastic on the wall. No attempt was made to control access to these global cues. The location of the experimenter varied across individual trials, moving to different locations relative to the arms of the maze. However, due to, the nature of the setup, namely goal arms being perpendicular to the edge of the bench, the experimenter was constrained to the right side (with respect to the training start box) of the maze.

Shoals
Twenty shoals of five fish were used and received all their training and testing as shoals. Training was completed 17/03/2014 and 17/04/2014. In a similar fashion to that of individual training. Again training would start at approximately 9 am and continue until all shoals had completed a full block. During training trials, half of the shoals in each group would receive a food reward in the left hand arm of the maze, and the other half in the right. In these instances, a trial would be considered complete when all five fish were in either the left or the right arm of the maze at the same time. This was the only difference in the training of individual fish and shoals. As with individuals, if the shoal swam to their allocated rewarded arm, the shoal would immediately receive a bloodworm reward administered with tweezers. If, however, the shoal swam to the other arm, they would receive the same two-minute “time out”. As with individual fish, a training block was considered successful if 8 out of 10 trials were correct and the shoal was
considered to have reached criterion when 3 consecutive training blocks were achieved.

**Shoals tested as individuals**

To assess whether individuals trained as a shoal displayed a different distribution of navigational choices compared to individuals trained individually, a second group of 20 shoals of five fish were also trained following the same procedure used in the training of the first batch of shoals. These experiments were completed between 19/08/2014 and 26/11/2014. As above, training started at 9 am and continued until all shoals had completed a full block of trials. The only difference was that upon reaching criterion and being tested with probe trials, these shoals were tested as the five individual members rather than as a shoal (see below).

**Probe trial - Individuals**

On reaching criterion, an individual would immediately receive a probe trial. This trial would begin in the opposite arm from training (Fig. 1), with the original start arm now blocked. Again a trial would be considered complete when the tail of the fish had passed into either the left or right hand arm. If the individual moved to the previous rewarded arm, this was recorded as a place strategy. If it swam using the same turning direction as on training trials (i.e. the opposite location to where it was rewarded), it was recorded as a response strategy. No reward was administered during probe trials. After the probe trial, the animal would be returned to their housing tanks and experimentation for that animal would be complete.

**Probe trial - Shoals**

As for individuals, for the first 20 shoals, on reaching criterion, a shoal would immediately receive a probe trial, in which they would start in the opposite arm from training with the original start arm blocked. Again, a trial would be considered complete on the first occasion that all five fish were either in the left or right hand arm at the same time. If the shoal moved to the previous rewarded arm, this was recorded as a place strategy. If the shoals swam using the same turning direction as on training trials (i.e. the opposite location to where it was rewarded), it was recorded as a response strategy. Again no reward was administered for probe trials. After the probe trial, the animals would be returned to their housing tanks and experimentation for those animals would be complete.

**Probe trial - Individuals trained as shoals**

A second set of 20 shoals was involved in the experiment to assess the navigational choice used by individual fish following training as members of shoals. Upon reaching training criterion, the shoal was immediately removed from the experimental tank and was placed in a beaker containing water from the experimental tank. A single fish would be placed into the probe start arm and would complete the probe trial alone with its choice recorded before it was removed and returned to its housing tank. As per the previous two groups, no reward was administered during probe trials. The water would be disturbed and allowed to settle before another fish would be placed into the start arm to complete the probe trial, and so on until all five fish had completed a probe trial.

**Shoal cohesion**

To assess whether all individuals within shoals made the same decision we noted whether a shoal was cohesive or non cohesive. Cohesive was defined as all fish entering the same goal arm on their first choice, whereas non cohesive was defined as the shoal splitting in their first choice of goal arm.

Due to a technical issue, only 18 of 20 probe trial videos were available for this analysis.

**Statistical analyses**

Data were analysed using SPSS statistical package (v20.0). A generalized linear model with an underlying poisson loglinear distribution was used to assess the effect of experimental group on the number of blocks required to reach training criterion. This performs better than a square root transformation and analysis assuming a Gaussian distribution [37]. Individual binomial tests were used to assess whether there was a significant preference for either a place or a response strategy on navigation choices made in each experimental group, and to assess the prevalence of shoal cohesion on the probe trial.

**Results**

**Acquisition time**

Acquisition time was the number of blocks required to reach training criterion by each shoal or individual fish (Fig. 2). There was a significant main effect of experimental group on task acquisition time: \(\chi^2\) (df = 2) = 54.15; \(P < .001\). Posthoc analyses showed that individual zebrafish took significantly more blocks to learn the training task than either set of shoals (\(P < .001\)). There was no significant difference between the two sets of shoals on acquisition time (\(P = .290\)). (See Fig. 3).

**Navigational strategy**

Figure 4 shows the percentage number of times in each experimental group that the fish adopted a place or response strategy during the probe trial. Individual
binomial tests showed that the experimental group that was trained and tested on the probe trial in shoals had a significant preference for choosing a place strategy (binomial test: \(N = 20, P = .012\)) whereas the group of fish who completed training and probe trials as individuals, and the group that completed training trials as shoals but the probe trial as individuals showed no significant preference for either a place or response strategy (binomial test: \(N = 20, P = .507\) and \(N = 100, P = .271\) respectively).

There was no significant difference in the time taken to reach a decision on the probe trial for each group. As the variance differed between groups (Levin's test: \(W^2 = 3.57, p = 0.031\)), the Welch test was used (ANOVA, Welch test, \(F = 0.94, p = 0.399\)).

**Shoal cohesion**

Individuals within shoals were significantly more likely to choose the same side as all shoal mates, i.e. remain cohesive than to choose different sides i.e. be non cohesive during their first choice of side on the probe trial; (Fig. 5, Binomial test: \(N = 18, P = .008\)). There was no relationship between cohesion and strategy choice (Fig. 6), with a place strategy dominating in both cases.

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**Fig. 2** Mean learning performance of each of the experimental groups during training. Numbers next to symbols indicate the number of units comprising that data point. The dashed line indicates the learning criterion.

**Fig. 3** Boxplot showing task acquisition time for each experimental group where “Individual” represents the group of 20 individual zebrafish that formed part of a previous study. “Shoal” was the group that were trained and received probe trials as shoals, and “Individual Probe/Shoal Training” indicates the group that received all training trials as shoals but completed probe trials as individuals. Rectangular boxes display 25th & 75th quartiles and the median. In both the “Shoal” and the “Individual Probe/Shoal Training” groups the median was equal to the minimum value of 3. Whiskers represent the 10th and 90th percentile of the data. Outliers outside this range are marked with circles.

**Fig. 4** Bar Chart showing the percentage number of times individual/shoals in each experimental group adopted either a place or a response strategy. An asterisk (*) indicates a significant difference between the two strategies for that group (\(P < 0.05\)). Numbers above bars indicate the n for that group.

**Fig. 5** Number of shoals observed to be cohesive and non-cohesive on the probe trials during the second set of experiments completed. Data was available for 18 out of 20 shoals only.
Discussion

This study investigated the effect of shoaling on the adoption of a navigation strategy in zebrafish (Danio rerio). Results showed that fish who completed the task in shoals took a shorter period of time to learn than individual fish. Unlike individuals, when tested on the crucial probe trial, shoals adopted a more consistent navigational strategy across the population with a significant preference for the allocentric “place” strategy being shown. As a result, shoals were more likely than chance to adopt a navigational strategy that took them to the location of the food reward in training when tested from a novel start point. These findings suggest that whereas individual zebrafish do not show a consistent navigation strategy, to learn the location of a food reward as a shoal, the most flexible navigation strategy is adopted from a novel start point (i.e. the one that takes them to the location of the food reward that was learned in training). Some caution is warranted in the fact that the fish from the individual experiments came from a different commercial supplier than those used in the shoaling experiments. A number of factors such as age, sex, rearing environment and nutritional status prior to housing were unknown. However, the consistency between individual responses on probe trials whether trained as an individual or as a shoal would suggest that the responses seen here are robust.

Many experiments, including this study, use a binary choice which places navigational decisions in conflict with group cohesion and thus may confound our understanding of the factors involved in collective decision making [38]. However, in the case of our experiment there was no a priori reason to expect that individuals that had learned a place strategy would be more likely to influence the group decision than those that had learned a response strategy. How this mechanism occurs requires further investigation. Place learning is a common phenomenon across a number of vertebrate taxonomic groups and appears to be the preferred strategy [39], but the preference for a place strategy is also influenced by a number of factors including age and quantity of training [14, 18]. One possibility is that if there are, on average, slightly more “place” learners than “response” learners within each group, then the majority may win, with the response learners sacrificing individual learning to maintain cohesion within the group. This is suggested by the fact that in this current experiment, more shoals than predicted by chance stayed cohesive, i.e. all chose the same goal arm as their first choice. However, further experimentation is possibly required, in which the number of place learners and response learners is controlled for, to explore this in more depth. Another possibility is that more dominant individuals use a place strategy, and thus those leading the shoal are more likely to choose place. Given that age is a factor in place learning, this remains an alternative explanation that also needs further investigation.

Zebrafish trained as shoals did not show a significant preference for either a place or a response strategy when tested from a novel start point as individuals. Whether this is because of the passenger effect, where following others overshadowed learning in some individuals, or because individuals within the group learn and maintain individual strategies in the training task, cannot be disentangled in the current study. A recent study on homing pigeons navigating as a flock has, however, suggested that leader-follower relationships may not only occur naturally, but may be inevitable in moving groups [23]. The study also suggests that those individual animals that take on the leadership role show more consistent and effective spatial learning when travelling alone, suggesting that, in some animals, individual learning may have an impact on the overall movement of the group. In spite of this, the results found in the study presented here do suggest that the more flexible information that determines the strategy of the shoal is not consistently transferred to individual zebrafish. This is perhaps not surprising as the training task could be solved equally successfully by either a place or a response strategy and so there appears no conceivable mechanism by which those using a place strategy could demonstrate this to others in the group.

As a shoaling species zebrafish are more likely to move around their environment in a group [34–36] and this has been argued to be beneficial for navigation (e.g. through the “many wrongs principle”) [40]. The results presented here confirm that living socially has an impact on the cognitive performance of a group of fish. First, it took individual fish significantly more time to reach training criterion, suggesting that moving in isolation.
has an effect on the learning abilities of this species. It is possible that having the animals from this social species complete the task alone could have caused stress. No visible signs of stress were observed, however, and all fish ate the food reward administered on correct trials – with avoidance of food being a common sign of stress in fish [41].

Another issue to take into consideration is the wall at the end of one arm of the maze. This wall could have provided a significant salient cue meaning that the “place” learning in this experiment may have been due to beaconing rather than the use of allocentric processes. If this was the case, it might have been expected that individuals would also show a significant preference for learning location, however neither individuals trained alone, nor those trained as shoals but tested alone, showed a significant preference for either strategy. This suggests that completing trials alone did affect spatial memory. A similar argument could be made for the restriction on which side of the maze the experimenter had to stand. Again, further investigation is required to fully assess what cues could have been used by the animals in this experiment. Previous studies have tried to do this by moving or altering the position of particular landmarks, to investigate which cues animals adhere to most when moving through a maze, or by totally eliminating landmarks [42–47]. Furthermore, the telencephalon has been identified as the area of the brain in fish responsible for encoding allocentric spatial information [48]. The same experimental design could be used again with ablated fish to assess whether such subjects would then show a preference for the response strategy. This was, however, beyond the scope of the current experiment.

Conclusions
This study demonstrates that moving in groups may cause individuals with different navigation strategies to converge on using just one strategy. In this case, it was the more flexible allocentric strategy that allows location of a goal from a novel start point that was used more often by shoals of fish. Whether this was due to do a majority rule, or a consequence of leader follower relationships through the choice of dominant individuals in the group, remains to be seen. Many species of fish show that shoal membership is fluid and is subject to fusion-fission on a regular basis [49]. Some fish species known to shoal have been shown to spend more than half of their time moving in isolation from conspecifics [49]. Because individual zebrafish do not appear to learn and transfer all relevant knowledge gained as a shoal member when navigating alone, this may indicate that individuals are at much greater risk alone than when in shoals as spatial memory is crucial for foraging and also for avoiding predators [50, 51].

Additional file

Additional file 1: File format: xlsx. Raw data file. This file contains the raw data that was used in the analysis of this paper. (XLSX 219 kb)

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Availability of data and materials
All data are included in Additional file 1.

Authors’ contributions
CLM, CMC and RAH designed the experiments. CLM carried out the experiments. CLM and RH analysed the data. CLM, CMC and RAH wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All experiments were completed in line with the guidelines for the treatment of animals in behavioural research and teaching [52]. Laboratory and experimental conditions were assessed by the Veterinary Services Division of the DHSSPS, Northern Ireland who deemed a home office project licence was not required for these experiments and was thus approved by the School of Biological Sciences ethics committee.

Consent for publication
Not applicable.

Competing interests
Richard Holland is an Associate Editor of BMC Zoology.

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