Symbolic quantitative cognition in wild zebrafish (*Danio rerio*)

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Abstract

Zebrafish (*Danio rerio*) constitute an excellent model system to investigate the neural and genetic basis of quantitative cognition because of the single neuron resolution of calcium imaging of awake, behaving fish. While nonsymbolic numerical cognition has been investigated across many taxa, symbolic numerical cognition has not been investigated among fish. We developed a novel quantitative symbolic test for zebrafish using an operant conditioning paradigm in which the number of horizontal lines zebrafish approached in a 2-alternative forced choice task predicted the number of food reward pellets they would receive. Zebrafish did not at the population level learn a preference for the 2-line stimulus predictive of receiving 2 food pellets. However, they performed significantly above chance in a nonsymbolic discrimination task with the same apparatus, in which the 2-line stimulus was associated with the same reward but the choice of the 1-line stimulus was not rewarded. We also explored the explanatory value of alternative spatial learning hypotheses such as a Win-Stay, Lose-Shift (WSLS) strategy at the individual level for fish in navigating these spatially randomised tasks. The implications of this for symbolic versus nonsymbolic quantitative cognition in this model system are discussed relative to reward type and stimulus modality.

Introduction

Humans are capable of a diverse set of mathematical operations, from the recognition of abstract numerical quantities even in the absence of explicit teaching, to learned symbolic numerical representation of quantities, mathematical operations upon numbers and even understanding ideas at the frontiers of modern abstract mathematics\(^1\,\,\,^3\). However, our understanding of the neuronal basis of human mathematical cognition is limited by the low resolution of human imaging, and animal model systems are necessary to take forward a circuit-level understanding of numerical and quantitative cognition. Of these various aspects of human numerical cognition, the skill most widely tested in non-human animals is non-symbolic numerical discrimination, through tasks involving spontaneous or learned discrimination between sets of objects of varying cardinal numerosity. **Our study examines symbolic quantitative cognition in a wild zebrafish model system**, which as a model system lends itself to large scale brain imaging with single cell resolution, as well as a strong genetic manipulation toolkit, both powerful tools to understand the neural basis of quantitative cognition.

Spontaneous quantity preference tasks have been insightful about the ecological value of general quantitative cognition and magnitude estimation, if not numerically specific cognitive skills. Quantitative cognition can be useful in gauging food quantity during foraging in chimpanzees\(^4\),
mice\textsuperscript{5}, cats\textsuperscript{6}, robins\textsuperscript{7}, crows\textsuperscript{8}, guppies\textsuperscript{9}, spiders\textsuperscript{10} and carnivores more generally\textsuperscript{11} in avoiding large groups of predators in redshanks\textsuperscript{12}, minnows\textsuperscript{13}, piranhas\textsuperscript{14}, guppies\textsuperscript{9,15,16}, kilifish\textsuperscript{17} or even persistent harassment from sexually interested conspecific males in mosquitofish\textsuperscript{18,19}, in assessing group size during social or sexual aggregation in monkeys\textsuperscript{20}, guppies\textsuperscript{21,22} and other poeciliids\textsuperscript{23}, mosquitofish\textsuperscript{18,19,24}, angelfish\textsuperscript{25} swordtails\textsuperscript{26}, sticklebacks\textsuperscript{27,28}, perch\textsuperscript{29}, zebrafish\textsuperscript{30–32}, mealworm beetles\textsuperscript{33} or during conflict in chimpanzees\textsuperscript{34}, lions\textsuperscript{35}, dogs\textsuperscript{36}, or even contexts such as parental care in cichlids\textsuperscript{37} and searching for shelter in snails\textsuperscript{38}. Most of these tasks deal with the animal’s ability to discriminate between the cardinality of two or more quantitatively specific stimulus sets, but ordinal cognition can also be useful for navigation. Experiments manipulating the ordinality of landmarks passed relative to distance passed suggest that ordinal information may be used instead of spatial distance by either a small fraction of individuals in some systems like honeybees\textsuperscript{39}, or by a majority of individuals for example in chicks\textsuperscript{40,41} and guppies\textsuperscript{42}, or along with spatial information in other systems like zebrafish\textsuperscript{43}.

Associative reward-based learning tasks that reward the choice of stimuli of a particular cardinality have been used successfully to elicit responses in a range of animals: chimpanzees\textsuperscript{44–54}, rhesus macaques\textsuperscript{4,55–57}, dogs\textsuperscript{58–62}, dolphins\textsuperscript{63,64}, racoons\textsuperscript{65}, rats\textsuperscript{66–70}, mice\textsuperscript{71–73}, horses\textsuperscript{74}, bears\textsuperscript{75} and birds like grey parrots\textsuperscript{76–79}, crows, pigeons\textsuperscript{80–87}, robins\textsuperscript{7,88}, canaries\textsuperscript{89}, and chicks\textsuperscript{40,41,90–92}. Work on other vertebrate systems such as reptiles: tortoises\textsuperscript{93} and lizards\textsuperscript{94} and amphibians: frogs\textsuperscript{95}, salamanders\textsuperscript{96} has been scarce, but there has been a mushrooming of work on numerical cognition and associative learning in fish: zebrafish\textsuperscript{31,97}, angelfish\textsuperscript{98–100}, archerfish\textsuperscript{101}, goldfish\textsuperscript{102}, redtail splitfins\textsuperscript{103}, mosquitofish\textsuperscript{104}, guppies\textsuperscript{105–107}, as well as some comparative work\textsuperscript{108–110}. Among invertebrate systems, bees have demonstrated impressive numerical abilities in associative learning tasks\textsuperscript{111,112}, including the use of numerical operations like addition and subtraction\textsuperscript{111}, which up to that point were only observed in rats\textsuperscript{66,67}, and chimpanzees\textsuperscript{44}.

Eliciting responses specific to the cardinal numerosity of stimuli suffers from the problem of numerosity correlating with various other attributes of the stimulus such as stimulus magnitude/ cumulative intensity, density, and convex hull. Associative learning studies controlling sequentially across trials for these correlates of stimulus numerosity demonstrated that the spontaneous or learned discrimination between sets of different cardinality may in some cases be based on stimulus magnitude rather than numerosity\textsuperscript{19,96,110,113–117}. Careful behavioural studies may also help resolve the role of the various mechanistic models proposed to underlie non-symbolic numerosity: a parallel individuation system (PIS)/object file system (OFS) to encode precise numerical quantities within the subitizing range - only up to and including the number 3\textsuperscript{118,119}; and for larger numbers, an approximate number system (ANS) to represent the numerosity of items in a set with a degree of error that increases with larger numbers\textsuperscript{3,120–122}. The existence of the PIS is also contested by the claim that the ANS system could also account for the entire range of numbers\textsuperscript{7,123}. An approximate magnitude system (AMS) has been proposed as a more
parsimonious interpretation of the data in non-human animals than the ANS, based on the claim that sequentially controlling for traits correlated with numerosity does not eliminate the possibility that a combination of those traits is used in place of numerosity for quantitative estimation\textsuperscript{124}.

Symbolic numerical representation has also been proposed as an aspect of numerical cognition that may enable precise numerical encoding even for larger numerosities in humans, and this has only been studied in a few other species like chimpanzees\textsuperscript{45,49,125–127}, monkeys\textsuperscript{128}, parrots\textsuperscript{78}, pigeons\textsuperscript{129}, bees\textsuperscript{130} besides humans\textsuperscript{3}, in comparison to the large body of work on non-symbolic numerosity.

Work on the neural basis of numerical cognition has shed light on the mechanisms involved in processing numerosity, identifying neurons responsive to stimulus numerosity in monkeys and crows\textsuperscript{131–136}, and areas active during numerical tasks in humans\textsuperscript{137–139}. However, the full circuitry involved in processing numerical information is best probed in a system such as zebrafish with a wide range of brain-wide calcium imaging\textsuperscript{140} and genetic tools\textsuperscript{141}. Initial molecular assays of c-fos and egr-1 expression have already narrowed down parts of the zebrafish brain that might be involved\textsuperscript{142,143}.

This study aims to examine the symbolic representation of reward quantity in wild adult zebrafish system, building upon experiments demonstrating the use of zebrafish as a genetically tractable full-brain imaging system showing non-symbolic numerosity\textsuperscript{43,97,109}. We were specifically interested in using symbols which could be extended to easily graspable manipulations with larger numbers: roman numerals I and II symbolizing the number of food pellets received as reward after choosing the corresponding symbol, in a T-maze based associative learning task.

**Results**

A) 

B)
Experiment 1: Associative learning of symbols predictive of food reward quantity

When we trained zebrafish to associate visual stimuli consisting of 1 or 2 symbolic lines, predictive of 1 or 2 food pellets respectively that would follow as reward, aggregating across all fish that completed the task (n = 28), we found no statistically significant learning of a first choice preference for the 2-line stimulus in this symbolic quantitative association task at the population level. A Shapiro-Wilk test conducted on the distribution of performance across fish for each day of 5 trials did not uphold the approximation of the proportion of choices by a normal distribution. The median proportion of trials per day (out of 5) across fish in which they chose the 2-line stimulus associated with two pellets of food was 0.6 +/- 0.2 (median +/- interquartile range) on day 1 and 0.4 +/- 0.35 on day 21 (median +/- interquartile range), and this median proportion did not increase beyond this range at any point in the 21 day period (Figure 2A).

A Kruskal-Wallis test shows that the proportions of daily trials in which fish choose the side with 2 lines (indicating two pellets of food) are not the same across days for all the fish combined (p-value = 0.04105), due to the high variation in the performances of the fish across individual days. But when comparing the performance across groups of consecutive days, using pairwise
Wilcoxon test results for a range of bins across groups of days, we found no significant learning at a population level in this task.

While the median proportion of trials in which the fish chose the 2-line stimulus began at 0.5 +/- 0.2 on day 1 of learning and stayed that way up to 21 days of assessment, indicating that the fish did not as a group reach the learning criterion of departing significantly from chance (0.5) for two or more consecutive training sessions.

Figure 2: The (a) per day and (b) cumulative proportion of trials in which zebrafish chose to move to the side with the visual stimulus with 2 lines and then receive 2 pellets of food, across 21 days (n=28).

**Individual results and alternative hypotheses**

Initial trials did not distinguish between individual fish, although we observed considerable interindividual differences, consistent with the literature which finds that half or even substantially more individuals among zebrafish do not reach learning criteria for these tasks. The population trend was also compared with batch-specific results and no departure from population-level results were found (data not shown). In the last batch of fish tested, the individual identities of fish were kept separate throughout trials, and interindividual differences were noted.

For all individually tracked fish, we explored alternative hypotheses to explain their behaviour. One such alternative hypothesis is that fish are side-biased rather than showing associative learning. If fish were to choose a single side of the maze to move to consistently, with the side-randomized presentation of stimulus they should end up making the more rewarded choice with respect to the visual stimuli 50% of the time. We plotted each individual fish’s performance with respect to the magnitude of the stimulus (which predicts reward magnitude) (thick red continuous line, Figure 3), alongside its choice of just the right side of the T maze (orange dotted
lines), across days of training (Figure 3). In parallel, we explored another hypothesis: that fish were utilizing a Win-Stay/Lose-Shift (WSLS) strategy with respect to spatial learning from past rewarded trials. Since the fish encounters a fluctuating reward relative to any given side, this hypothesis posits that fish continue to choose a particular side if it is rewarded in the previous trial, but would switch sides in the absence of reward. We calculated an extension of this WSLS scoring metric (plotted in blue dotted lines, Figure 3) where the fish’s behaviour is given a score of 1 if the fish returns to the last visited side and received the higher reward with 2 pellets of food, OR if it avoids the last visited under rewarded side with 1 pellet. The fish gets a score of 0 if it visits the same side as before despite getting a lower reward in the previous trial, or if it avoids the last visited side even if that led to a higher reward.

Figure 3: The proportion of trials for individual fish with better (A, B) and worse (C, D) performance in choosing the side with 2 lines in the 1 vs 2 food reward paradigm (thick red continuous line), plotted alongside the proportion of trials in which they chose just the right side (orange dotted lines), and the average Win-Stay/Lose-Shift scoring metric (blue dotted lines) across days of training.
In later trials of the 2 vs 1 reward paradigm where 8 fish were tested individually, we found inter-individual differences not only in performance on the task, but in side bias and in the Win-Stay/Lose-Shift score of their choices. Fish F1 seems to initially learn a right-side bias and later chose the 2-line stimulus (Figure 3A); while fish M4’s rise in the proportion of trials choosing the 2-line stimulus over time can be explained both by a Win-Stay/Lose-Shift strategy and a left side bias (Figure 3B). In F3’s case, the fish uses a right-side biased approach relatively consistently after the first two days of the experiment, with a relatively low score in learning the association between stimulus and reward quantity (Figure 3C). A combination of the side bias and WSLS score explains the fish M3’s choices from trial to trial better than the fish learning a symbolic association between the stimulus and the reward quantity it represents (Figure 3D). We contend that these alternative hypotheses have not been explored in other numerical cognition experiments.

**Experiment 2: Associating quantitative symbols with presence/absence of a food reward**

Given that the zebrafish did not show statistically significant performance in the association task between quantitative symbols and a quantity of reward at the population level, and our protocol differed on several counts from the protocols used in the literature to assess quantitative cognition: utilizing locally wild-caught zebrafish, a T-maze and computerised horizontal line stimuli, we attempted to replicate a simpler task documented in the zebrafish literature to ensure that we could draw conclusions about their performance on this task rather than just about our setup.

Using the same set up, we assessed the performance of zebrafish in learning to choose the side of the tank displaying the 2-line stimulus, with a simpler reward structure: the 2-line stimulus was rewarded with 2 pieces of food, and the 1-line stimulus with an absence of food reward (2 vs 0 reward), rather than with the number of lines being associated with the quantity of the food reward (2 vs 1 reward).

The proportion of trials in which fish chose the 2-line stimulus associated with the presence of reward was 0.4 +/- 0.2 (median +/- interquartile range) on day 1 and 0.8 +/- 0.00 on day 21 (median +/- interquartile range). At the population level an elevation in learning is seen by the 14th day (Figure 4B) with a further enhancement in performance and reduction in performance variability in the last few days (days 26-30) (Figure 4A). One sample Wilcoxon signed-rank test showed that the population level proportion of choosing the side with the 2-line stimulus for this days 26-30 is significantly higher than chance level (0.5) (p-value = 0.0002406).
Figure 4: The proportion of trials in which zebrafish choose the side with 2 lines to then receive a reward in the presence/absence of food reward paradigm across days of training (n=5) (A) per day and (B) cumulative proportion of trials across 29 days of training (n=5).

A Kruskal-Wallis test shows that the proportions of choosing the side with line 2 are not the same for all the fish combined across all the days (p-value = 0.03804).
Figure 5: The proportion of trials in which fish choose the side with 2 lines in the presence/absence of food reward paradigm (dark red continuous line), compared with the proportion of trials in which the right side was chosen (orange dotted lines), and the Win-Stay/Lose-Shift scoring metric (blue dotted lines) for individual fish with better (A, B, C, D) and worse (E) performance across days of training.

Therefore, in line with the literature\textsuperscript{97,109}, significant associative learning does occur in this task among a subset (n=5) of fish tested (n=12), while the remaining fish tested showed freezing or frantic movement in response to the stimulus or considerable side bias in their responses within the first few days of training. Since all fish tested in this experiment were individually tracked, we could compare their side bias and WSLS score alongside their choices between the two visual stimuli (data for all five fish shown in Figure 5). Of the five fish who did not show freezing or consistent side bias, D1, D3, D5 and D7 are examples of individuals who showed an improvement in the proportion of choosing the side with the visual symbol of two lines over the days (Figure 5 B, C and E). Fish D4 is an example of an individual who did not show any improvement in the proportion of choosing the side with line 2 (Figure 5).

These results are interesting because the WSLS spatial learning score is higher than the associative learning performance curve in the individual analysis for all the fish as and when they show increasing scores for the associative learning task, and whose results are pooled together in the population level learning in Figure 4. This suggests that zebrafish may perform well in an associative learning task based on a spatial learning strategy, despite the experimental paradigm involving stimulus side randomization.

Discussion

The interpretation of experimental results in numerical and quantitative cognition tasks in animals has always been difficult due to the difficulty controlling the many variables correlated with the numerosity of the stimulus. Our study attempts to overcome this barrier partly by
presenting visual stimuli that bear numerosity as symbols, without an attempt to control for other information bearing correlates of that visual stimulus, and to investigate the abilities of zebrafish to learn an association between these symbols and the numerosity/magnitude of reward they predict. This is therefore a first attempt to investigate associative quantitative learning of a visual predictor of reward quantity in wild zebrafish - where both visual stimulus and food reward bear numerosity along with magnitude, and the stimulus functions as a symbol with predictive value for the associated reward quantity. Given what is known about nonsymbolic numerosity in zebrafish, the investigation of symbolic quantitative cognition is a next step necessary to examine the ability of zebrafish to perform numerical operations, in order to understand the cognitive abilities of a model system in which calcium imaging can cast a wide net across the brain in examining the neural basis of quantitative abilities as well as the genetic underpinnings of clinical variations such as dyscalculia.

Our results show that at a whole population level, zebrafish did not learn to move towards the symbol associated with the larger number of food pellets. We consider two alternative explanations for this. The most banal is that zebrafish were not strongly enough motivated by food - some fish had to be excluded from the study after a couple of days of training because they did not always eat the food pellet - or that these results are specific to our experimental setup involving wild zebrafish, horizontal line stimuli, and a T maze. A T-maze may be more cognitively challenging than compared to the single tank setup used in past studies on zebrafish cardinality, but we employed a T-maze because zebrafish have been successfully trained to learn to associate a food reward with colour-based cues in a T-maze and with visual and spatial cues in a plus shaped maze.

We addressed the question of our results being specific to our setup by testing the same fish on a simpler task of learning the association of the same symbolic numerical stimuli with the presence or absence of the food reward. We found that the zebrafish performed similarly to the findings of Bisazza and Santaca and better than the findings of Agrillo et al., with half the fish reaching the learning criterion. The fish that reached learning criteria in our setup showed significant learning of the discrimination task with the presence/absence of reward, suggesting that our experimental setup and food motivation alone cannot explain our negative results on the symbolic cognition task. Therefore, our study suggests that zebrafish may not be able to learn the association between these visual symbols and the different quantities of food reward they predict.

Zebrafish might be able to perform a quantitative symbolic learning task that is structured differently. Zebrafish can spontaneously use ordinal cues even when they are placed in contradiction with spatial cues after being trained on mixed cues for a food reward in some tasks,
although this behaviour is also task type specific - they use both ordinal and spatial cues for other tasks\textsuperscript{43}. On the other hand, while zebrafish have been shown to spontaneously discriminate the cardinality of different group sizes of conspecific fish for sexual assortment based on past memories of group size\textsuperscript{31} and shoaling\textsuperscript{32} respectively, these studies did not control for continuous magnitude information (Potrich et al, 2015\textsuperscript{31}) and olfactory information (Seguin and Gerlai, 2017\textsuperscript{32}) respectively. In fact, multiple fish species, including zebrafish, have been shown to spontaneously use continuous magnitude information in shoal size choice, with no numerosity preference once overall surface area and magnitude are equalized between shoals of different cardinalities\textsuperscript{110}. The first assessment of the ability of adult zebrafish to associatively learn to discriminate between groups of abstract visual shape stimuli of different cardinalities, that controlled for the correlates of numerosity, was part of a comparative study across teleost fish species\textsuperscript{109}. This inter-species work indicated that far fewer zebrafish reached the learning criteria than the other species tested (guppies, mosquitofish, angelfish, redtail splitfin), and also performed worse than redtail splitfin at a control task involving associating geometric shape discrimination with a reward\textsuperscript{109}. This, along with Gatto et al.\textsuperscript{149} suggests that the problem with zebrafish may be not with quantitative cognition in particular but with visual shape discrimination for certain kinds of visual stimuli. If so, this would impact performance in any quantitative learning task involving sets of visual stimuli, including our own. However, a recent set of work contests both these findings\textsuperscript{109}, suggesting that zebrafish perform well\textsuperscript{97}, and equivalently to other fish (guppies\textsuperscript{150}) in numerical tasks as well as in tasks involving visual shape discrimination\textsuperscript{151}. While Bisazza and Santaca\textsuperscript{97} do not attempt to explain the difference much, methodological differences may explain differing outcomes in numerosity experiments on a given model system\textsuperscript{152,153}. The salience of the visual stimuli used may also impact outcomes\textsuperscript{149} - Bisazza and Santaca\textsuperscript{97} used simpler and more ecologically relevant circular visual stimuli than Agrillo et al.\textsuperscript{109}, who used a variety of abstract shapes. In fact, it was this consideration that prompted us to use simple lines of the kind seen in Roman numerals rather than the more abstract symbolic stimuli used by Agrillo et al.\textsuperscript{109}, or Arabic numerals. We used lines rather than circles to ensure the fish could see both stimuli while making a choice at the stem of the T maze. If we had found successful symbolic association in this task, our next step would have involved the use of more abstract symbols, and to reverse the association between these two symbols and the quantity of food they predict, to check whether the visual stimuli are being understood symbolically rather than as nonsymbolic indicators of magnitude.

The considerable inter-individual variability documented between individual zebrafish in terms of reaching the learning criteria in associative learning tasks in some studies\textsuperscript{109} but not in Bisazza and Santaca\textsuperscript{97}, was also found in our experiments where we tracked individuals, as well as in several other fish behaviour studies\textsuperscript{22,151,154–158}. An additional factor in our case is that the fish we
used are wild type zebrafish from the rivers in India, which is their native habitat\cite{159,160}, and high
degree of variation in learning and memory behaviours have been demonstrated across different
wild type populations of zebrafish\cite{161}. It is important to bear in mind that different labs working
on this question may be working with strains derived from different wild populations, or with
strains whose cognitive abilities have diverged considerably from the wild populations they were
sourced from\cite{162,163}, while dealing with differences in results across labs (Bisazza and Sancata\cite{97}
versus Agrillo et al.\cite{109}). The other possible factor playing into these inter-strain and inter-
individual differences might be varying ecologically specific advantages of quantitative and
numerical cognitive abilities.

Despite reports of inter-individual differences, most work on quantitative discrimination or
numerical cognition in fish has largely reported only population level results and have not
explored spatial learning hypotheses\cite{31,104,109,151,164,165}. Our study and others in fish\cite{155}
and other model systems\cite{136,166,167} all indicate that individual data could provide valuable insights which
could be missed if studies on numerical tasks in fish ignore heterogeneity within the responses of
a population. One of the inter-individual differences that emerged related to the degree to which
fish were biased towards one side of the T maze, and this is why we explored a spatial learning
hypothesis of ‘Win-Stay/Lose-Shift’, where fish make the decision based on the association of
spatial cues with the latest positive reward, to see if it fit the data. The criticism\cite{124} of the strategy
of inferring the perception of numerosity from experiments that sequentially randomize various
parameters correlated with numerosity across trials, keeping numerosity constant, also applies to
inferring associative learning of stimulus type from tasks where the spatial presentation of the
stimulus is randomised across trials. In both cases, sequential randomisation does not preclude
the possibility that the animal uses a complex strategy (for example, WSLS in our case) related
to the sequentially controlled parameter (in our case, spatial location).

Results of the simpler visual discrimination task we conducted show that zebrafish at a
population level are able to associate each of two symbolic visual stimuli with the presence/
absence of a food reward respectively, demonstrating that our use of wild zebrafish and a T maze
with an appetitive conditioning protocol aren’t responsible for the failure of our fish to perform
at the symbolic cognition task. However, our fish were trained for a longer time than most
zebrafish associated learning tasks reported in the literature\cite{104,109,148,161,168}. Most studies stop
training when the fish perform significantly higher than chance level, but we followed a more
stringent criterion of waiting for that performance to last for multiple consecutive days due to the
inter-day variation in performance (as per \cite{43,97}). Even though zebrafish in our study showed
significant learning from day 14, we extended the experiment and found that over time, the
median performance in doing the task which had reached 0.6 (the performance documented in
increased further and reached and stabilised at 0.7 (as documented in 97) along with reduced variability. Prolonged training can therefore elevate the performance in associative learning tasks in zebrafish. The question of whether prolonged training can elevate performance was also raised by Bisazza and Sancata\textsuperscript{97} in comparing the relatively low accuracy in results of training fish, with experiments involving thousands of trials in birds and mammals where the accuracy is closer to 90-95%; and in fact, such extensive training has been shown to raise the performance of goldfish beyond 90% with 1200 trials\textsuperscript{102, 169}.

This work could be taken forward by examining symbolic quantitative cognition in zebrafish using other testing paradigms involving social reward, which can sometimes be more potent than food rewards\textsuperscript{9,170}. Given the general performance of zebrafish on visual shape discrimination, symbolic quantitative cognition could be tested using other modalities such as auditory stimuli. It is also clear that all quantitative learning results that use spatial randomization must be considered in the light of alternate hypotheses such as spatial side bias and spatial win-stay lose-shift strategies: further experimentation would be necessary to examine zebrafish associative learning tasks to distinguish between these two hypotheses explaining their results. We have also used wild type zebrafish caught from their natural habitats in India and it would be valuable to conduct a comparative study looking at the population and/or strain specificity in these quantitative cognitive abilities such as has been conducted on learning and memory\textsuperscript{161}. Finally, work on this model system raises the possibility of using neuroimaging and genetic techniques to understand the neural and genetic basis of non-symbolic and symbolic quantitative cognition abilities and disabilities.

**Methods**

**Subjects**

Experimental subjects were wild-caught zebrafish (\textit{Danio rerio}) bought commercially and in turn sourced from local ponds and rivers in India. The first batches of 4+6+7+3 zebrafish, used for experiments in 2017 spring, 2017 fall, 2018 spring and 2018 summer respectively, were sourced from a local commercial pet shop in Chittaranjan Park, New Delhi, India. A second set of 11 zebrafish were purchased from a pet shop in Sonipat, Haryana, and the third batch of 8 fish was sourced from a local pet store in Daryaganj, New Delhi, India, and these were used for experiments in the fall of 2021. All of these fish were experimentally naive.

The first batches were housed in a generic cuboid glass aquarium (18x12x9 inches), and filled with 20 litres of RO-purified water, for at least 7 days before the experimental period began. The water in the tank was filtered through a SOBO® WP-950F 6-Watt sponge filter and
the tank was maintained at 27 °C (± 1 °C) using an RSElectrical® RS008-A 50-Watt submersible water heater. The second and third batches of fish were housed in the ZebTec Active Blue - Stand Alone system (Tecniplast, PA, USA), which was auto-maintained a pH of 7.50-8.50, 28-30°C temperature and 650-700 µS conductivity. In all housing conditions, the fish were maintained in a 14:10 light:dark photoperiod by switching-on and off a Roxin® RX-300 6-Watt fluorescent LED providing ambient light using a Smaerteef® programmable power-strip, and later using a custom-built switch that automatically switched on and off the light at the designated time intervals. The subjects were maintained one a daily feed of Tetra® Tertabits Complete flake food, except during the training phase when they did not receive food outside the training paradigm. All appropriate international, national and/or institutional guidelines for the care and use of animals were followed.

Apparatus
For training and testing, a transparent glass T-Maze was purpose built for the experiment (Figure 1). The dimensions of the stem were 20x4x15 inches (LxWxH) while the dimensions of the arms in total were 20x4x15 inches (LxWxH), with each arm’s dimensions being 8x4x15 inches (LxWxH). A sliding gate was fixed 5 inches up the stem made of transparent polycarbonate material attached to the tank with epoxy-resin adhesive (Araldite®). For stimulus presentation, a BenQ® Zowie XL2411 24-inch 144 Hz 1080p monitor was located right in front of the maze arms and connected via HDMI to a Lenovo® S400z All-in-one PC. In order to record experimental trials for later analysis, a Nikon® Coolpix A900 was mounted via a Manfrotto® 290 dual tripod over the T-maze. The maze contained 5 litres of RO-filtered water, with the water level maintained just above the position of the stimuli on the screen. In order to provide a controlled visual environment and block visibility of the experimenters, 24x36 inch white chart paper was used to cover the maze setup.

Stimuli
The stimuli were produced by a custom written MathWorks® MATLAB script using Psychtoolbox. The stimuli consisted of black (RBG 000) 6-pixel thick horizontal lines drawn on an all-white background (RGB 256 256 256) in the bottom third of the screen. The presentation of the stimuli with sets of two and one lines on either side of the screen was randomly chosen in the MATLAB script.

Testing protocol: associating numerical symbols with food reward pellet quantity
The experimental protocol was divided into 3 phases – the acclimatization phase (3 days), the training phase (20 days) and the testing phase (the last and 21st day). Each day of training involved a sequence of 10 rewarded trials for the first three batches of fish, and 5 trials for the
last batch. No difference was found in results between the batches. The final testing day involved the same number of unrewarded probe trials.

For acclimatization the fish were transferred to the T maze using a small cup such that the fish stayed within water with no periods of asphyxiation. Our pilot trials showed initial fear responses in zebrafish when newly introduced to the T maze, either freezing or frantic swimming, and we adapted the acclimatization protocol in Sison and Gerlai\textsuperscript{148} to habituate the fish to the maze in decreasing-size shoals over three days before the first training session. On day 1, 4 fish were transferred together to the T-maze, sample stimuli were put up on the screen and 10 Tetra\textsuperscript{®} Tertabits Complete food flakes were dropped at the ends of each T-maze arm at both ends of the left and right arm. The fish were allowed 2 hours to acclimatize together and get familiar with both the structure of the maze and the location of food. This was repeated with pairs of fish on day 2, and then for individual fish on the third day.

For training and testing sessions, individual fish were transferred into the T-maze for a 20 minute acclimatization period, after which the MATLAB stimulus script was triggered, displaying the stimuli consisting of 1 and 2 horizontal lines located randomly on either side of the screen. Then, the gate was lifted and after the fish made a first choice towards one arm of the maze, the fish was rewarded with either one food flake or two, depending on the stimulus displayed in the arm it chose. As soon as the fish consumed the reward, the stimuli were replaced with a blank white screen. For the second and third batch of fish, additional gates were closed after the fish made the first choice to ensure the reward was eaten - this made no difference to the results (analysis not shown). All sessions were videotaped and analyzed by a second observer who was blind to the side-stimulus association.

**Testing protocol: Associating numerical symbols with a food reward**

We implemented another version of our experimental protocol as a control, for a simpler task that has been studied and reported in the literature. This tested associative learning of the same visual stimuli, but instead of the number of stimulus lines predicting the number of food flakes received as reward, there was a 2 pellet reward for the larger stimulus and no reward for the smaller stimulus. The protocol was otherwise identical to the one above.

For the task of associating visual stimuli with food reward, a sample size of \(N=7\) was calculated as the number of individual fish required to obtain a significant effect similar to literature which conducted a similar task with zebrafish\textsuperscript{31,43}. This number was obtained by conducting a priori power analysis using the program G*power 3.1.9.4\textsuperscript{171} using the average effect size observed in
literature for similar tasks in zebrafish\textsuperscript{31,43}. The power analysis was conducted for the two-tailed Wilcoxon signed-rank test with power $(1 - \beta)$ set at 0.80, effect size $(d) = 1.489$ and alpha = 0.05.

**Statistical Methods**

All statistical tests were conducted in R v.1.2.2. A Shapiro-Wilk test was conducted to check if our data were derived from a normal distribution. To check for any differences in the proportion of fish choosing the side with 2 lines across days of training in the 1 vs 2 food reward paradigm, we conducted a Kruskal-Wallis test. In order to check for any consistent change in the proportion of trials in which the fish chose the side with 2 lines, and to pool across days to reduce inter-day stochasticity, we conducted a Kruskal-Wallis test across non-overlapping sets of 5 consecutive days. We conducted pairwise Wilcoxon tests with Bonferroni correction to test the significance of the differences in the proportion of fish choosing the side with 2 lines between groups of 5 consecutive days. We used a One sample Wilcoxon signed rank test to check for the significance of the performance of the fish in the last set of 5 consecutive days above chance level (0.5).

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**Author Contributions**

The study was designed by BKR and DS; the apparatus was made by DS and GS; the manuscript was written up by NAM and BKR; data collection and initial data analysis was performed individually by DS and NAM, and by four teams consisting of NK, RS, AW, SM, AS, SK, NS; RM, SS, NR, YS; ABG, MB, MS, TB, LB, AM, ST, AB, NW; data processing and further analysis was performed by NAM, DS, ABG, TB, SH, IB, AM, NK.

**Data Availability**

All raw data files are uploaded at this link: https://osf.io/xhk7u/?view_only=8150b31c011b41cb9965f4e4daf06719

**Competing Interest**

The authors declare no competing interest.
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