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Low Repeatability of Aversive Learning in Zebrafish (*Danio rerio*)

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– review and editing, Supervision. **Hamza Anwer:** Writing – review and editing. **Rose O’Dea:** Data Curation, Writing – review and editing, Visualisation. **Daniel Hesselson:** Resources, Writing – review and editing. **Shinichi Nakagawa:** Conceptualization, Methodology, Software, Formal Analysis, Resources, Data Curation, Writing – review and editing, Supervision, Funding acquisition

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Summary Statement

Zebrafish were trained to avoid colour stimuli through negative associations with a mild electric shock. Very low repeatability estimates suggest individuals were not distinguishable by their aversive learning abilities.

Abstract

Aversive learning – avoiding certain situations based on negative experiences – can profoundly increase fitness in animal species, yet no studies have systematically quantified its repeatability. Therefore, we assessed the repeatability of aversive learning by conditioning approximately 100 zebrafish (*Danio rerio*) to avoid a colour cue associated with a mild electric shock. Across eight different colour conditions zebrafish did not show consistent individual differences in aversive learning ($R = 0.04$). Within conditions, when zebrafish were conditioned to the same colour, blue conditioning was more repeatable than green conditioning ($R = 0.15$ and $R = 0.02$). Overall, aversive learning responses of zebrafish were weak and variable. We speculate that the effect of aversive learning might have been too weak to quantify consistent individual differences, or directional selection might have eroded additive genetic variance. We also discuss how confounded repeatability assays and publication bias could have inflated estimates of repeatability in the literature.

Introduction

Animals use the cognitive process of learning, which can be defined as a change in behaviour due to past experience, to respond to the environment (Kawecki, 2010). Learning has a profound influence on survival and reproductive success (Krebs & Davies, 1987; Skinner, 1984), and has been studied in a wide range of taxa. For example, individual learning speed has been correlated with foraging performance in bees (Raine & Chittka, 2008) and grasshoppers (Pasquier & Grüter, 2016); and greater cognitive capacity has been linked to higher reproductive success in magpies (Ashton et al., 2018) and male robins (Shaw et al., 2019), as well as to healthier body condition in wild primates (Huebner et al., 2018).

Animals learn through association, which is reinforced differently by positive and negative experiences (appetitive and aversive learning, respectively). Appetitive learning takes place when individuals associate a stimulus with a 'positive' event, usually a food reward stimulus, whereas in aversive learning the association is with a 'negative' event, usually a fear inducing stimulus. Failing to learn from positive experiences (appetitive learning) prevents a potential benefit (i.e., a minor opportunity cost). Failing to learn from negative experiences may yield an immediate fatal cost. Therefore, both types of learning can increase lifetime fitness and drive natural selection, but appetitive learning may be under weaker selection than aversive learning.

For traits to evolve they need heritable variation that can be subject to selection. For labile traits (i.e., traits expressed more than once over a lifetime) the consistency of individual differences in trait expression indicates potential heritability. The common approach to quantify consistent individual differences in eco-evolutionary studies is estimating the statistical index 'repeatability' (*R*; otherwise known as the 'intra-class correlation coefficient' or ICC; Lessells & Boag, 1987; Nakagawa & Schielzeth, 2010). Repeatability partitions variance into within-individual (residual) and between-individual components. Biologically, the repeatability of a trait indicates the amount of observed variance which is due to individuals sustaining trait differences between each other (Nakagawa & Schielzeth, 2010), but estimates can be inflated by measurement errors and experimental confounds (Dohm, 2002; Niemelä & Dingemanse, 2017).

Generally, behavioural traits are moderately repeatable (R = 0.34; Bell et al., 2009; cf. Holtmann et al., 2017), with cognitive behavioural traits showing somewhat lower repeatability (R = 0.15 - 0.28; Cauchoix et al. 2018). Our understanding of how natural selection shapes the evolution of cognitive traits remains poor (Boogert et al., 2018). Despite the extensive literature on aversive learning, no published study has comprehensively quantified its repeatability (but note Cauchoix et al. (2018) includes three unpublished studies with some measures of aversive learning). To reduce this knowledge gap, we quantify the repeatability of aversive learning behaviour in zebrafish (*Danio rerio*), a popular model organism in cognitive science (Gerlai, 2016; Norton & Bally-Cuif, 2010). Zebrafish exhibit a range of distinct behaviours that can be measured in previously established assays (Fangmeier et al., 2018; Meshalkina et al., 2017).

Here, we use an avoidance conditioning assay — associating a visual cue with a mild electric shock (see Fig. 1A-E) — to thoroughly assess the repeatability of colour preferences and aversive learning in both male and female zebrafish. We expect individuals to consistently differ in their aversive learning speeds (i.e., separation of better and worse learners), but do not have particular expectations for sex differences. We estimate repeatabilities in two different ways. First, we examine repeatability across different colour pairs (four different pairs with eight possible combinations: 8 measurements per individual; Fig. 1F). Given the estimates for appetitive learning summarised in Cauchoix et al. (2018), we predict a low to moderate repeatability. Second, we test whether repeatability is increased in a constant learning environment by using just one colour pair (both combinations of green and blue; 3 repeated measurements per individual for each colour; Fig. 1F). For both types of repeatability measurements (within and across learning environment), we also quantify colour preferences and their repeatabilities, to give a comparator in individual differences that can be compared to aversive learning.

Materials and Methods

Zebrafish population

Adult wildtype zebrafish were bred on the 24th of January 2019 (5 months old at the commencement of experiments) and maintained at the Garvan Institute of Medical Research in Sydney, Australia. The wildtype stock was derived from of a mixture of Tübingen long fin, AB and other unidentified strains to increase genetic diversity, which had been interbred for 8-10 generations. Fish were housed in 3.5L Tecniplast ZebTEC tanks (maximum of 24 fish per 3.5L tank) under standard laboratory conditions (~28°C; ~pH 7.5; ~1000 µs conductivity; 12/12h from 7:30 light/dark rotation) and fed live *Artemia salina* nauplii twice a day and commercially available fish food once per day (O.range GROW-L).

We marked juvenile fish for individual identification at around 90 days post-fertilisation with coloured tags (red, brown, purple, black, white, yellow, orange, pink, or green). For marking, fish were anesthetised in a tricaine solution (4.2ml of 0.4% in 100ml of system water) for 20 seconds before being injected with Visible Implant Elastomer tags (VIE, Northwest Marine

Technologies, Inc.; Shaw Island, Washington, United States). We injected fish twice (unless one mark was blank), one on either side of the dorsal fin (Hohn & Petrie-Hanson, 2013). Among these marked fish, we used a total of 103 zebrafish with approximately equal sex ratios kept in 4 tanks of 24 individuals (12 males, 12 females) for both experiments. At any one time during the experiments, the same 96 fish were used, but to compensate for death, illness or experimenter error, seven fish were replaced by seven new fish over the three-month study. Due to incomplete data for zebrafish size (described below) the across conditions and within conditions analyses included 93 and 94 zebrafish, respectively. The Garvan Animal Ethics Committee approved all procedures described above and experiments described below (ARA 18_18). Further, Garvan veterinarians oversaw fish welfare associated with aversive learning prior to our pilot tests.

Experimental Design

Aversive Learning Assay

We used an avoidance conditioning method to quantify aversive learning in a simple, automated assay (Brock et al., 2017; Fontana et al., 2019). We ran all assays using four Zantiks AD units (Zantiks Ltd., Cambridge, UK; see https://osf.io/t95v3/ for further details). The units employed infrared tracking using an integrated computer to record fish movement and collect data. In the assay, a visual cue (colour or pattern) was associated with a negative stimulus (brief mild electric shock; 7V DC 80ms), which motivated fish to avoid the associated visual cue. We then measured the extent of avoidance (i.e., time spent away from the cue associated with an electric shock) compared to the baseline preference to quantify aversive learning (learning response). We based our initial assay parameters (e.g., the acclimation period, voltage, etc) on previous research (Brock et al., 2017), and subsequently modified the parameters based on the outcomes of pilot tests.

Before each assay we individually placed fish into one of four lanes within rectangular tanks (see Fig. 1A). For the assay, we exposed the fish to four stages; (i) Habituation: we habituated the fish to isolation in a novel environment over a 30-minute acclimation period (Fig. 1B); (ii) Baseline: the tank was visually split into two even zones via the colour displaying screen at the bottom of the tank (Fig. 1C). One of these two colours would later become conditioned with the mild electric shock (CS+), the other colour remained unconditioned (CS-). Here, the position of the colours (left or right) automatically switched every five minutes for a 30-minute period, and we recorded zebrafish preference for the CS+ to obtain a baseline preference before conditioning; (iii) Conditioning: first, the CS+ (visual cue associated with shock) was displayed across the entire screen for 1.5 seconds then immediately afterwards paired with the US (mild electric shock) to condition the fish to an aversive experience. Second, the CS- (visual cue not associated with shock) covered the screen for 8.5 seconds (Fig. 1D). This phase was repeated nine times, sufficient for fish learning to avoid the CS+; and (iv) Probe: akin to the baseline period, the tank was split into two even zones (left or right) depicted by different visual cues. We tracked fish movement and recorded fish preference for the visual cue associated with the shock (CS+) over 5 minutes. During this time, the visual cues switched every minute (see Fig. 1E). We used only 2 minutes out of the 5-minute probe time since we determined a clear decrease in learning response in our observations. This probe length is similar to other studies: Brock et al. (2017) used a 2-minute probe, and Fontana et al. (2019) used a 1 minute probe. Probe CS+ preference was used in comparison to baseline CS+ preferences to quantify learning.

Experimental Conditions

We used a range of colour conditions to test aversive learning. Each condition was comprised of two visual cues, one aversive and one control (CS+ paired with CS-) (Fig. 1F). We selected different colour combinations to use as visual cues for the zebrafish, which had either been worked in pre-existing assays or were reported to evoke a clear colour preference (Brock et al., 2017; Roy et al., 2019). As a result, we chose seven colours (green, blue, grey, orange, magenta, red, yellow) and 1 pattern (check; hereafter, this pattern is also referred to as a 'colour' with the others). We used four visual cue combinations ('Check/Grey', 'Green/Blue', 'Red/Yellow', 'Magenta/Orange') and their reverse ('Grey/Check', 'Blue/Green', 'Yellow/Red', 'Orange/Magenta') for a total of eight conditions. For example, the 'Check/Grey' condition used check pattern as the CS+ (cue associated with shock) and grey colour as the CS- (control cue); the 'Grey/Check' condition used grey colour as the CS+ and check pattern as the CS-, and so on.

Prior to the experiment, we assigned fish into quartets (four fish that underwent trials within the same unit/assay tank simultaneously) that systematically rotated between trials. The balanced design accounted for three potential confounding variables: the time of day (quartet rotated), Zantiks unit (quartet rotated), and lane position (individual within quartet rotated). We estimated repeatability in two different situations (across conditions and within a single condition). Across conditions, we ensured fish experienced trials from all four colour pairs before subjecting them to their exact reverse four conditions (with trials conducted over four weeks in June and July 2019). We included this form of reverse learning to negate memory of the CS+ colour between trials, which may impact both baseline and probe colour preference. Within conditions, each zebrafish underwent trials in the 'Blue/Green' and 'Green/Blue' conditions a further two times (over two weeks in September 2019).

Fish Size Measuring

We took photos of each fish approximately one week after across-conditions trials and another set of photos approximately one week after within-conditions trials. We captured top down photos of live fish and measured fish in ImageJ (Schindelin et al., 2015). We used fish length (standard length) and width (at widest part of body) to calculate the ellipsoid size of the fish by using $\pi\left(\left(\frac{fish\ length}{2}\right)\times\left(\frac{fish\ width}{2}\right)\right)$. This controlled for a potential size effect resulting from loss of penetrance and effectiveness of the mild electric shock due to larger body size.

Data Processing and Analysis

All data processing and analyses were conducted in the *R* computing environment (version 4.0.2; R Core Team, 2019). Linear mixed models were run using the *lme4* package (version 1.1.21; Bates et al., 2014) in conjunction with the lmerTest package (version 3.1.2; Kuznetsova, Brockhoff, & Christensen, 2017) that provides Satterthwaite's degrees of freedom correction. We obtained repeatability values via the *rptR* package (version 0.9.22; Stoffel et al., 2017) that uses the *lme4* pacakge to run mixed models. Based on visual assessments of residual distributions, assumptions of normality and constant variance were not clearly violated. The Zantiks units recorded time spent in each CS zone, total distance travelled and how often fish changed zones. All code, and the raw and processed data, are available at: https://osf.io/t95v3/. We deemed our results statistically significant at the alpha = 0.05 level (or when 95% confidence intervals did not overlap zero).

Quantifying Aversive Learning

We determined learning by the difference in time that fish spent in the CS+ before and after the aversive experience. To analyse learning across all the sessions included in this study, we used the time difference (‘*difference’ = time spent in the CS+ during baseline – time spent in the CS+ during probe)* as the response variable in a linear mixed-effects model (LMM) via the *lmer* function in the *lme4* package. We fitted individual ‘fish ID’ (96 levels) and ‘experimental condition ID’ (8 levels, see Fig. 1F) as random effects in the model. Also, we included the following fixed effects: (1) ‘sex’ (female or male) to investigate sex differences in learning, (2) ‘day’ since first trial, to account for time effects of sequential days on learning or learning via repeated trials (e.g., 1 being the first day and 8 being the 7th day from the first), (3) ‘fish size’ to control for fish’s response to conditioning which might be size dependent due to potential differences in body penetrance of a mild shock, (4) ‘learning’ (initial and reverse) to find if learning was affected when the CS± of a condition were switched in successive trials. Note that we *z*-transformed the fixed effects ‘day’ and ‘fish size’ to make the intercept meaningful and slope estimates comparable (Schielzeth, 2010).

Quantifying the Repeatability of Aversive Learning

We obtained enhanced agreement repeatability (hereafter referred to as repeatability) estimates by incorporating statistically significant fixed effects from the model and retaining their variance in the denominator (Nakagawa & Schielzeth, 2010). We only fitted the random effect ‘fish ID’ and included ‘sex’ as a fixed effect. The R package *rptR* computes repeatability values using the within and between individual variance in linear mixed models fitted with restricted maximum likelihoods (Nakagawa & Schielzeth, 2010). Using *rptR*, we obtained standard errors and 95% confidence intervals (CIs) through parametric bootstrapping, with each model set to 10,000 bootstrap samples. Following Bell (2009) and

Wolak (2012), we categorised our repeatability results into low (<0.2), moderate (>0.2 - <0.4) and high (>0.4).

Colour Preference and Repeatability

An underlying assumption of our aversive learning assay was that zebrafish can discriminate between different colours. Therefore, from the baseline period (prior to aversive conditioning), we quantified underlying colour preferences (tendency to associate more heavily with one colour in a pair), and the consistency of individual differences in colour preference (i.e., repeatability of colour preference).

In each condition, preference for one colour was only compared to the other paired colour (e.g., preference for red is only relative to preference for yellow; see Fig. 1F). Given we examined relative colour preference, preferences for either colour in a condition were the inverses of each other. Hence, to be able to determine colour preference for each colour, we grouped conditions of matching colours into four groups for analysis (e.g., Group 1, 'Red/Yellow' & 'Yellow/Red'; Group 2, 'Green/Blue' & 'Blue/Green'; Group 3, 'Check/Grey' & 'Grey/Check'; Group 4, 'Orange/Magenta' & 'Magenta/Orange').

To analyse relative colour preference, we ran LMMs for each group of colours using across conditions data. We used baseline colour preference as the response variable 'baseline' for these models. We fitted the random effect 'fish ID' in the models (Group 1 & 4, 97 levels; Group 2 & 3, 98 levels; levels differ because one fish died prior to completing all conditions). Further, we fitted the following fixed effects: (1) 'day' (days since first trial) to control for potential colour preference change with time, (2) 'sex' (male or female) to account for sex differences and (3) 'learning' (initial and reverse) to see the effect of reverse learning on colour preference. To determine the repeatability of colour preference, we used *rptR* mixed-effects models with the response variable ‘baseline’ to generate repeatability estimates. We did not find any fixed effects to be statistically significant, as such, they were excluded, and the colour preference models were fit with the random effect ‘fish ID’.

Results & Discussion

We found negligible repeatability in aversive learning across the eight different conditions / colours (*R* = 0.04, 95% CI [0.001 - 0.097], Fig. 2A), despite individuals being able to discriminate between colours (as measured by moderate to high repeatabilities for colour preferences; Grey: *R* = 0.45, 95% CI [0.276 - 0.607]; Green: *R* = 0.45, 95% CI [0.278 - 0.604]; Red: *R* = 0.43, 95% CI [0.250 - 0.584]; Orange: *R* = 0.46; 95% CI [0.283 - 0.605] Fig. 2B). Within conditions, we found very low repeatability in one condition (‘Green/Blue’ Fig. 2A; *R* = 0.02, 95% CI [0 - 0.153]), and low repeatability in the other (‘Blue/Green’ Fig. 2A; *R* = 0.15, 95% CI [0.023 - 0.278]). Therefore, the substantial variation in aversive learning we observed (as in Fig. 3A) was most likely driven by current (intrinsic or extrinsic) environmental factors, rather than additive genetic variance or canalized developmental differences (cf. Sznajder, Sabelis, & Egas, 2012).

Zebrafish showed strong relative colour preference in all four conditions (see Fig. 3B). We found preference for grey, green, red and orange, over check, blue, yellow and magenta, respectively. The strongest relative colour preference was found for red and orange, providing further evidence that zebrafish prefer colours with longer wavelengths (Roy et al., 2019). We did not find any statistically significant sex difference in colour preference, aversive learning, and their repeatability estimates (see Supplementary Information Table S1-2 and Fig. S1). Of relevance, a previous meta-analysis of repeatability for behavioural traits found males tend to be more repeatable than females (Bell et al., 2009), but reported this result to be inconclusive.

Our findings of low repeatability for aversive learning are surprising, given low to moderate repeatability of behaviour and cognition reported in two meta-analyses. For general behaviour, Bell et al. (2009) reported an average repeatability of $R = 0.34$. For cognitive performance, Cauchoix et al. (2018) found $R = 0.15\text{-}0.28$, mostly based on temporal repeatability estimates from appetitive learning trials. Below we discuss four potential reasons why zebrafish in our experiment showed much less consistent individual differences in aversive learning compared to the previous estimates from Cauchoix et al. (2018) and Bell et al. (2009).

First, while zebrafish did demonstrate aversive learning, the average effect was small, and in many trials, individuals did not seem to avoid the negative stimulus. On average, individuals spent just 4-6 fewer seconds per minute in the negatively associated colour following conditioning (across conditions: female average = 3.89 seconds per min, SE = 1.05, $t_{33} = 3.65$, $P < 0.001$; male average = 5.64 seconds per min, SE = 0.94, $t_{22} = 5.21$, $P < 0.001$; Fig. 3A). The small effect could be caused by individuals not learning or quickly forgetting. It is also possible that learning performance would be greater at the group level; zebrafish are a shoaling species and learning may have evolved to depend on group dynamics. When tested individually, zebrafish display more variable behaviour and are more prone to stress (Pagnussat et al., 2013). Low repeatability could therefore be caused by zebrafish being largely insensitive or unresponsive to the conditioning when housed individually (i.e., poor aversive learners, a weak assay or anxious fish with impeded movement). However, the fact that there was a population shift in the direction of aversive learning raises the question of why individuals who learnt in one trial did not maintain their performance across trials; if a particular subset of zebrafish had consistently learnt, or failed to learn, then we would have detected higher repeatability. Further, while the behaviour change following aversive conditioning was modest, zebrafish learnt much faster (in 1.5 minutes) compared to previous assays with appetitive training (e.g., over 20 days; Brock et al., 2017). As far as we are aware, no studies have investigated a relationship between the strength of associative learning and the magnitude of repeatability. Furthermore, it should be noted that our experiment only considered visual cues, but in the wild, fish often use chemical cues to detect danger (Brown, 2003). Although technically more challenging, aversive learning using different sensory cues other than visual cues should be considered in the future.

Second, past selection pressures on our study population may have eroded additive genetic variance associated with aversive learning, which was not restored in the intervening generations. In the wild, aversive learning could be under strong selection (e.g., to learn to evade predators), and individuals could be selected to learn from negative experiences as quickly as possible. Indeed, aversive learning could be under stronger selection than appetitive learning, as mortality costs of negative experiences can easily exceed opportunity costs of missing positive experiences. Stronger selective pressures could explain why we found substantially lower repeatability for aversive learning compared with previous results for appetitive learning. In a similar vein, traits more closely associated with fitness (e.g., aversive learning) tend to not be as heritable (thus, repeatable; cf. Dohm, 2002) than less fitness related traits (e.g., appetitive learning; Merilä & Sheldon, 2000). However, we cannot be sure that the performance of zebrafish in our laboratory assay accurately captures their ability to aversively learn in their natural habitat.

Third, some of the repeatability values in the meta-analyses by Cauchoix et al. (2018) and Bell et al. (2009) may have been overestimated. An inflated repeatability estimate, also known as ‘pseudo-repeatability’, is the result of within-individual variation being erroneously accredited to differences between individuals (Niemelä & Dingemanse, 2017; Westneat et al., 2011). Pseudo-repeatability occurs when the conditions between measurements are too similar (e.g., environmental conditions are unchanged or intervals between measurements are too short) and might explain why we found higher repeatability when zebrafish were measured repeatedly within a single condition (‘Blue/Green’; $R = 0.15$), compared to across eight separate conditions (although no inflation was seen in ‘Green/Blue’). Indeed, Cauchoix et al. (2018) and Bell et al. (2009) included studies with testing conditions which did not change over the course of a study, similar to our within-condition estimates. Further, most studies in both meta-analyses had relatively short intervals between measurements (most intervals were under a week in Cauchoix et al. (2018), and almost all were under a year in Bell et al. (2009). The short intervals between measurements reported in Bell et al. (2009) were significantly associated with higher repeatability values, consistent with pseudo-repeatability. Relevantly, two recent studies on birdsong reported that associative learning among individuals was not repeatable between years, indicating that estimates obtained over short intervals may not be a true reflection of consistent individual differences defined in animal personality (Soha et al., 2019; Zsebők et al., 2017).

Fourth, publication bias might have contributed to an inflation of the overall repeatability estimates in the published literature (cf. Parker et al., 2016). The average repeatability of 0.34 reporting by Bell et al. (2009) was based on a meta-analysis of published studies. Cauchoix et al. (2018) included many more unpublished datasets (n = 38) compared to published datasets (n = 6); they mentioned that their unpublished datasets produced, overall, a lower repeatability estimate than that of the published studies. This finding is consistent with the pattern that larger effect sizes are more likely to be published. Recent studies are increasingly reporting non-significant and low repeatability (e.g., Reichert et al., 2020; Vernouillet & Kelly, 2020). Therefore, an updated future meta-analysis may reveal a lower overall repeatability estimate in behaviour.

In conclusion, zebrafish did not show clear consistent between-individual differences in aversive learning. The low repeatability could potentially indicate that strong past selection pressure has almost driven aversive learning to fixation, because of the vital importance of learning to avoid danger. Alternatively, low repeatability may be due to the small effect of fish learning to avoid the stimuli. In addition, published repeatability estimates could be inflated by within-individual variance frequently being measured as between-individual differences (i.e., ‘pseudo-repeatability’), and by publication bias. We contend that these issues can be diminished in future behavioural research by controlling for confounding effects and reporting every estimate of behavioural traits, whether repeatable or not.

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Figure Legends

Figure 1

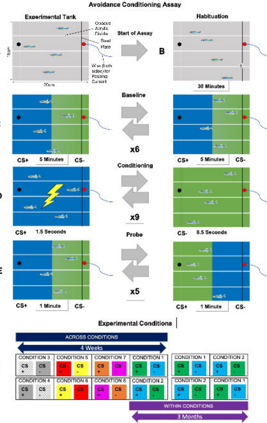
Colour conditions and aversive learning assay: (**A**) zebrafish are placed in the experimental tanks and (**B**) acclimated to the novel environment for 30-minutes; (**C**) in a 30-minute baseline period, initial CS± preference is established; (**D**) during the conditioning phase, fish are presented the CS+, then immediately subjected to a mild electric shock; and (**E**) in a 5-minute probe phase, learning is determined by fish spending less time in the CS+ when compared to the baseline. (**F**) Each condition is a combination of two visual cues (zones), one conditioned to a mild electric shock (CS+), the other is not (CS-). Across conditions eight colour conditions and eight sessions (each session is represented by a white box). Within conditions: two colour conditions and four sessions (in addition to two sessions in Experiment 1).

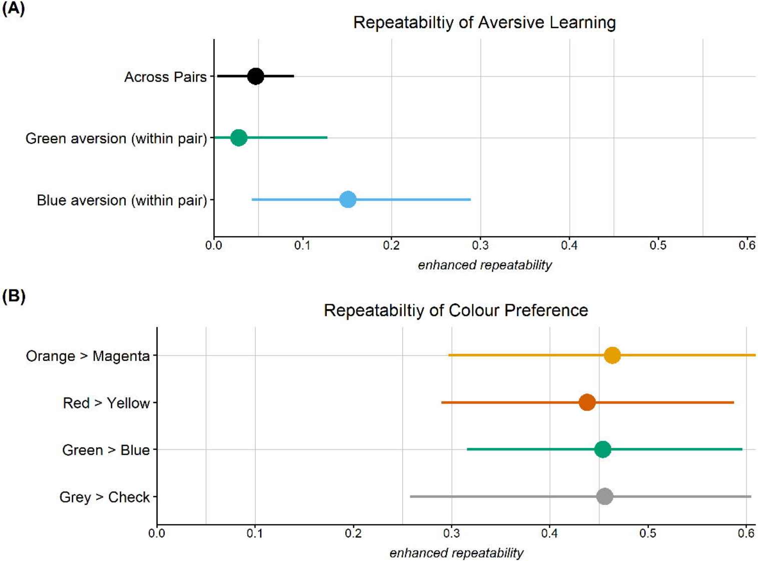
Figure 2

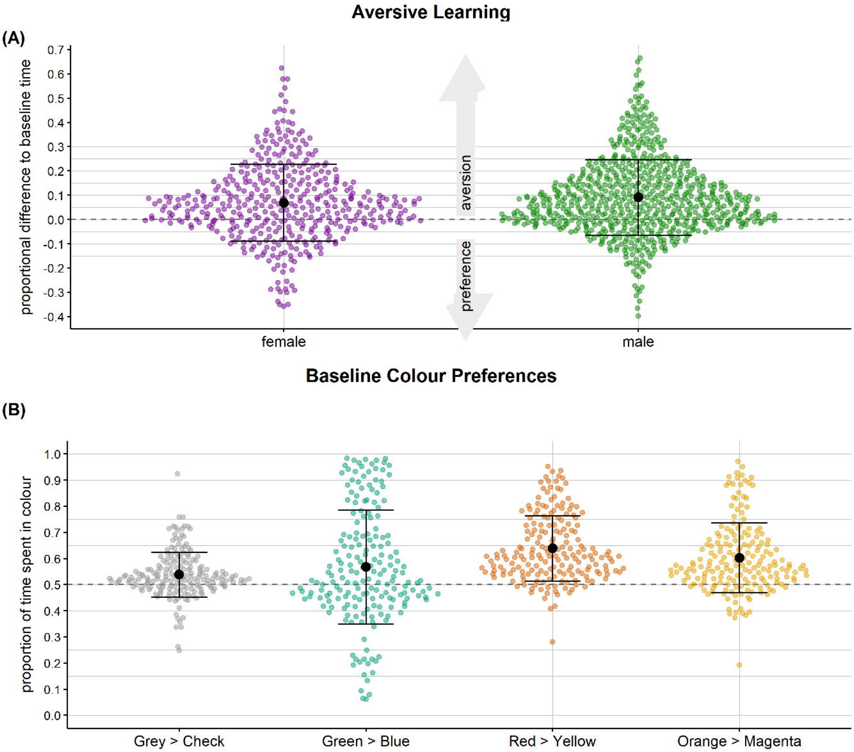
Repeatability of aversive learning and colour preference in zebrafish. Points and whiskers represent means and 95% confidence intervals via parametric bootstrapping. (**A**) Zebrafish show somewhat consistent individual differences in aversive learning within the 'Blue/Green' pair, but not within the 'Green/Blue' pair or across all colour combinations. (**B**) Zebrafish show consistent individual differences in colour preferences (variation depicted in Fig. 3B).

Figure 3

Violin plots for aversive learning and colour preferences. Smaller coloured points depict individual trials. Larger black points and error bars depict means and standard deviations of observations. (**A**) The top panel shows means and variation in aversive learning, split by sex (female = purple; male = green) when all the session data is combined. Points above the line at zero depict trials in which zebrafish spent less time in the aversive stimulus colour in the probe period (the colour associated with an electric shock) relative to the baseline period (i.e., aversive learning). (**B**) The bottom panel shows the tendency of zebrafish to favour one colour in a pair during the baseline period (i.e., before administration of electric shocks). The dashed horizontal line at 0.5 represents no colour preference (i.e., spending 30 seconds in each colour zone).


Avoidance Conditioning Assay
Experimental Tank
Opaque Acrylic Divider
Steel Plate
Wire (both sides) for Passing Current
15cm
20cm
A
Start of Assay
Habituation
B
30 Minutes
Baseline
C
x6
CS+
5 Minutes
CS-
CS+
5 Minutes
CS-
Conditioning
D
x9
CS+
1.5 Seconds
CS-
8.5 Seconds
Probe
E
x5
CS+
1 Minute
CS-
CS+
1 Minute
CS-
Experimental Conditions
ACROSS CONDITIONS
4 Weeks
F
CONDITION 3
CS +
CS -
CONDITION 5
CS +
CS -
CONDITION 7
CS +
CS -
CONDITION 1
CS +
CS -
CONDITION 1
CS +
CS -
CONDITION 2
CS +
CS -
CONDITION 4
CS +
CS -
CONDITION 6
CS +
CS -
CONDITION 8
CS +
CS -
CONDITION 2
CS +
CS -
CONDITION 2
CS +
CS -
CONDITION 1
CS +
CS -
WITHIN CONDITIONS
3 Months


(A)
Repeatabiltiy of Aversive Learning
Across Pairs
Green aversion (within pair)
Blue aversion (within pair)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
enhanced repeatability
(B)
Repeatabiltiy of Colour Preference
Orange > Magenta
Red > Yellow
Green > Blue
Grey > Check
0.0
0.1
0.2
0.3
0.4
0.5
0.6
enhanced repeatability


Aversive Learning
(A)
proportional difference to baseline time
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
aversion
preference
female
male
Baseline Colour Preferences
(B)
proportion of time spent in colour
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Grey > Check
Green > Blue
Red > Yellow
Orange > Magenta