# Visual modality modulates operant learning effect in larval zebrafish

(should not exceed 12, 000 words, including figure legends)

(Subdivision – numbered sections: why it would be like this

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.)

Contents

[Visual modality modulates operant learning effect in larval zebrafish 1](#_Toc516848800)

[**Abstract** 2](#_Toc516848801)

[**Introduction** 3](#_Toc516848802)

[**METHODS (avoid comment and discussion)** 4](#_Toc516848803)

[**1.** **Ethics statement** 4](#_Toc516848804)

[**2.** **Animals and housing** 4](#_Toc516848805)

[**3.** **Experimental Set-ups** 4](#_Toc516848806)

[**4.** **Software Suites (BLITZ and ABLITZER)** 5](#_Toc516848807)

[5. **Behavioral Experimental Procedure** 5](#_Toc516848808)

[6. Behavioral Analysis 5](#_Toc516848809)

[a) Turn analysis 5](#_Toc516848810)

[b) Shock analysis (Not to mention) 5](#_Toc516848811)

[c) Distance to centerline 5](#_Toc516848812)

[d) Extinction analysis 5](#_Toc516848813)

[**Results** 6](#_Toc516848814)

[1. Larval zebrafish showed significant learning effect in a visual operant learning task 6](#_Toc516848815)

[a. Fig.1 Experimental Diagram; 6](#_Toc516848816)

[b. Fig.1 Learning Paradigm; 6](#_Toc516848817)

[c. Fig.1 UniScatter Plot for each strain with red-black checkerboard as conditioned visual pattern; (Based on time and turn) 6](#_Toc516848818)

[d. Fig.1 Example traces for each strain (distance to the centerline)) (6-10dpf) 6](#_Toc516848819)

[2. Visual modality modulates operant learning effect in larval zebrafish 8](#_Toc516848820)

[a. Fig.2 Histogram plot that compares PItime and PIturn before and after training 8](#_Toc516848821)

[b. Fig.2 Example Traces (distance to midline for each case (pattern-strain pair) 8](#_Toc516848822)

[3. Trial-by-trial analysis reveals different subtypes of learner and non-learners in larvae 10](#_Toc516848823)

[a. Plot learning curve: learned, not learned, all (refer Jennifer Li.) 10](#_Toc516848824)

[b. Separate hist plot for learners and non-learners 10](#_Toc516848825)

[c. Self-abuse fish 10](#_Toc516848826)

[4. The relation between bias and learning effect 11](#_Toc516848827)

[5. Memory extinction in larval zebrafish 11](#_Toc516848828)

[a. Fig.5 Show memory extinction histogram for each pattern-strain pair 11](#_Toc516848829)

[b. Fig.5 Plot memory extinction versus fish age 11](#_Toc516848830)

[6. Ontogeny of operant learning in GCaMP for different conditioned pattern 11](#_Toc516848831)

[a. Fig.4 Learning percentage changes with fish age (6dpf-10dpf) 11](#_Toc516848832)

[7. Calcium imaging of electric shock in naïve larval zebrafish 11](#_Toc516848833)

[a. Set-up diagram 11](#_Toc516848834)

[b. Labeling 11](#_Toc516848835)

[c. Calcium changing curve 11](#_Toc516848836)

[8. Calcium imaging of conditioned response in learned larval zebrafish 11](#_Toc516848837)

[a. Experimental settings 11](#_Toc516848838)

[b. Labeling 11](#_Toc516848839)

[c. Calcium changing curve with visual stimulus 11](#_Toc516848840)

[9. GCamp fish learned better than WT fish and more sensitive to conditioned visual pattern. 11](#_Toc516848841)

[a. Fig.3 Compare learning effect in each case between WT and GCaMP fish 11](#_Toc516848842)

[b. Fig.3 Compare sensitivity (change between pure black case and red black checkerboard case) between WT and GCaMP fish 11](#_Toc516848843)

[**Discussion** 12](#_Toc516848844)

[**Acknowledgment (all references should be cited in the text and vice versa)** 12](#_Toc516848845)

[**Reference (similar to Harvard citation)** 12](#_Toc516848846)

# **Abstract**

Transparency and relatively small size have made larval zebrafish a promising model for understanding learning mechanism at whole brain scale. With a well-defined operant learning paradigm, we demonstrated fearing might play an important role in associative learning in larval zebrafish. The more frightening the conditioning pattern is, the better the larval zebrafish learn. In the following head fixed behavioral paradigm with simultaneous calcium imaging, we showed the close relation between fearing circuit and learning circuit in larval zebrafish.

We provide an end-to-end operant conditioning paradigm and data acquisition and analysis software suites.

# **Keywords**

# **Highlights**

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

# **Introduction**

Operant conditioning as a well-defined learning paradigm has played an important role in understanding underlying neural mechanisms in mammalian systems. However, it is difficult to monitor whole-brain neural activities during the entire learning process. Larval zebrafish is a promising vertebrate model for investigating the neural principles underlying learning whose transparency, small size and a great compromise between system complexity and practical simplicity enable behavioral recording with simultaneous whole brain calcium imaging during the entire learning process. Several groups have reported operant learning(Valente 2012,) or other associative learning paradigms(Erin Shuman,) in larval zebrafish, however, whether the learning efficiency depends on the stimulus modalities remains unknown. More importantly, though some learning-related brain regions have been identified, how unconditioned stimulus and conditioned stimulus are represented in the neural circuits is far from clear.

Here, we reported ….

# **Material and METHODS (avoid comment and discussion)**

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

* 1. **Ethical statement of animals-using (change it a little bit)**

All animal handling and care were conducted in strict accordance with the guidelines and regulations set forth by Chinese Academy of Sciences, University of Science and Technology of China (USTC) Animal Resources Center, and University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the USTC (permit number: USTCACUC1103013).

* 1. **Animals and raising**

Forty zebrafish (*Danio rerio*) of the genetype (huc:h2b-gcamp6f) were used in the experiments. All fish tested were from 7 to 10 dpf (day past fertilization) larvae. They were bred, raised and housed in the same environment. Fish were fed two times per day from 6 dpf with paramecium in the morning (8-9 A.M.) and evening (6.7 P.M.) until used in the experiments. The water was replaced with E2 medium (https://zebrafish.org/documents/protocols/pdf/Fish\_Nursery/E2\_solution.pdf) in the morning (8-9 A.M.) and evening (6.7 P.M.). The water temperature was maintained at 28.5 °C. 33% of Illumination was provided by fluorescent light tubes from the ceiling with lights turned on at 08:00 h and off at 22:00 h.

* 1. **Experimental Setup**

The behavioral experiment system with custom software suites and supported hardware was built to achieve an end-to-end high-throughput experimental workflow.

* + 1. Hardware

Zebrafish swam freely in custom-built acrylic containers with transparent bottoms. Each container is divided into four arenas separated by opaque walls. The arena’s size is 3 cm × 3 cm × 1 cm, with water filled. Each arena holds one fish at most in the experiment. 3 infrared-sensitive CMOS camera (Basler aca2000-165umNIR, Germany) cameras with adjustable lens (Canon, Japan. Model EF-S 18-55mm f/3.5-5.6 IS II) parallelly captured swimming behavior at ten frames per second. 3 infrared LED light sources (Kemai Vision, China, model HF-FX90, wavelength 940 nm) illuminate each container from the below. For each camera, a 700 nm long-pass filter (Thorlabs FEL0700, US.) is positioned in front of the camera to block visible light to facilitate online imaging processing of custom software BLITZ. Visual stimuli were presented by a projector from the top over all 3 containers (PIQS Projector S1, 14.6\*7.85\*1.75 cm, 854 × 480 pixels). Electric shocks (100 ms, 9 Volt/3 cm) were delivered via two platinum filament, one on each side of the arena. Shock delivery at each arena was programmingly controlled by custom software BLITZ via a 16-channels relay (HongFa JQC-3FF, China). Room temperature was controlled by an air-conditioner at 27 °C.

* + 1. Software Suites

Custom C++ software BLITZ (Behavioral Learning In The Zebrafish) with Microsoft Visual Studio 2017 processed 3 video streams in parallel to get real-time head, center, tail position and heading angle by using Pylon library (citation) and the open source OpenCV computer vision library (citation). The program also rendered visual pattern and control electrical shocks delivery based on timeline and fish motion parameters in real-time. All the important experimental information (Experimental context information, e.g. experiment start time; visual pattern index and shocks delivery information; fish motion parameters) were recorded in human- and computer- readable YAML files. Raw videos were recorded.

The BLITZ software is available at <https://github.com/Wenlab/BLITZ>.

Another custom MATLAB (The MathWorks, Inc.) software ABLITZER (Analyzer of BLITZ Results) was used to import YAML files, run behavioral analysis and statistical analysis and visual analytic results as figures.

The ABLITZER software is available at <https://github.com/Wenlab/ABLITZER>.

* 1. **Behavioral Experimental Procedure**

Fish were fed at least before using in the experiment. Fish were placed via a Pasteur pipette (Nest, US) from the raising tank to the experimental arenas. Behavioral experiment would not run until fish start moving around in the surface of the water to avoid inference of startle response to the novel context. For each fish in paired-group, it will be trained first with self-control protocol, then with operant learning protocol. For each fish in unpaired-group, it will be trained first with self-control protocol, then with unpaired operant learning protocol.

Fish used in paired-group and unpaired-group were all naive fish before the experiment.

* + 1. Operant learning protocol

In this protocol, fish will experience 3 different stages in order: 1. Baseline; 2. Training; 3. Test

First, in the 10 minutes baseline stage, the visual pattern beneath each arena would interchange between fig. s1.a (CS on the top) and fig. s1.b (CS on the bottom) with a random duration (uniformly sampled from 30 to 45 seconds) until the end.

Second, in the 20 minutes training stage, the visual pattern would update upon fish’s behavior. Also shocks delivery was also only dependent on fish’s behavior. After the visual pattern updated (including the first visual pattern in the training stage), fish has 7 seconds thinking time to escape from the CS area. If fish was on the CS area after the thinking time, whole-arena shocks would be delivered every 3 seconds until fish escape from the CS area. After a continuous staying in Non-CS area for 48 seconds, the visual pattern would update with equal chance to one of fig. s1.a and fig. s1.b. Then continue the above procedures until the end.

After the training stage, comes 1 minute blackout period to deprive all visual stimulus.

Finally, in the 18 minutes test stage, to test whether the fish built the association between the CS pattern and US shock, the visual pattern changes every two minutes from fig. s1.a to fig. s2.b until the end.

* + 1. Self-control protocol

Except no shocks were delivered (power supply off) at the training stage, everything keeps the same as the operant learning protocol.

* + 1. Unpaired operant learning protocol

All stages are the same as the operant learning protocol except the training stage.

In the training stage, 200 shocks (comparable with average shocks fish received in the operant learning protocol, give a number) were randomly given in 20 minutes to fish without pairing with visual pattern.

* 1. Behavioral Analysis
     1. Pre-Screening

Fish with data quality lower than 0.95 were excluded from the following analysis, since those fish do not act spontaneous swimming very often, in this way, those fish were considered not in good condition. Data quality = 1 - #bad frames/#frames. Bad frames are frames that fish freeze over 1 seconds and frames with image processing failure.

* + 1. Non CS area time proportion (time index)

Time Index = frames on the Non-CS area / frames in total

* + 1. Turning analysis (provide a diagram)

The analysis was adapted from Valente. 2012 with some modifications. Heading angle change between two consecutive frames exceeds 15 degrees would be counted as a turn. The fish would get +1 score when performing an escape turn and it will get -1 score when performing a stupid (change the word) turn. The escape turn is defined as fish on the Non-CS area turn back within 2 body length to the mid-line. The stupid turn is defined as fish on the CS area turn back within 2 body length to the mid-line.

The turning index = number of escape turns / number of escape turns and stupid turns.

Then normalize the turning index to [0,1] in accordance with time index.

* + 1. Distance to the mid-line

The distance is Euclidean distance from the fish head position to the mid-line with a sign. The sign is -1 when fish is on the CS area and 1 on the Non-CS area.

* + 1. Learning analysis

To evaluate whether fish learned the operant learning task or not, we divided the entire operant learning protocol time into 24 trials of 2 minutes in order. Since in the test stage, there is no shocks paired with CS, the memory might extinct. (give a reference). The extinction point is defined as the first time that the time index of a trial drops below the time index of the fish at the entire baseline stage. In this way, we defined the time from the start of the test stage to the extinction point as the retrievable period.

A fish is counted as a learner to the operant learning task when the time index in the retrievable period is significant higher than the time index in the baseline stage. (using unpaired t-test)

* 1. Statistical Analysis

t-test was used for self-control comparison and unpaired t-test was used for comparison between different fish.

# **Results**

## Larval zebrafish showed significant learning effect in a visual operant learning task

* 1. Fig.1 Experimental Diagram;
  2. Fig.1 Learning Paradigm;
  3. Fig.1 UniScatter Plot for each strain with red-black checkerboard as conditioned visual pattern; (Based on time and turn)
  4. Fig.1 Example traces for each strain (distance to the centerline)) (6-10dpf)





Introduce visual operant learning paradigm,

Introduce BLITZ system and ABLITZER software suites (refer Leifer’s paper)

Introduce learning metrics

Report significant learning effect

Example trace of a learned fish

In the operant learning paradigm, the conditioned stimulus (CS) red-black checkerboard pattern is paired with whole-tank moderate electric shock as the unconditioned stimulus (US). To accelerate the experimental progress and make the experiment more replicable, we developed a high-throughput end-to-end behavioral system and supported software suites BLITZ and ABLITZER (see methods). BLITZ provides a fully automated workflow from video capture, online image processing to visual stimulus presentation and electric shocks delivery. For the post-processing part, ABLITZER allows users to import, analyze and store experiment data in a well-structured way, furthermore, ABLITZER has plenty of visualization methods to represent data in a meaningful way. (Make it more concise)

With red-black checkerboard as conditioned stimulus pattern (CS) and moderate electric shock as the unconditioned stimulus (US) in a modified operant learning paradigm (see Methods), we surprisingly found that some of (give a percentage) 7-10 dpf zebrafish larvae are able to pair CS with the US and eventually learned to avoid staying CS area. Because larval zebrafish have the light preference, we set two kinds of control groups to investigate whether it is a fake (use better word) result or real learning effect. The first kind of control setting is no-shock operant learning protocol, in other words, everything is the same as normal operant learning procedure but no shocks given in the training session. The second kind of control setting is unpaired operant learning protocol, in other words, 200 shocks (which is comparable to the average shocks fish received in the normal operant learning protocol) are not paired with CS but randomly given in the training session. By comparing with two control settings, we found only the normal operant learning group showed significant learning effect.

The learning effect is quantified with two metrics – NCS time proportion and turning performance index. Those two metrics were also used in Valente 2012.

## Visual modality modulates operant learning effect in larval zebrafish

* 1. Fig.2 Histogram plot that compares PItime and PIturn before and after training
  2. Fig.2 Example Traces (distance to midline for each case (pattern-strain pair)









Since larval zebrafish has little preference to pure gray than red-black checkerboard, we wonder less contrasted pattern pair and more contrasted pattern pair would lead to any difference in learning effect. So we choose 1. white-black checkerboard as CS pattern and pure gray as NCS pattern. 2. Pure black as CS pattern and pure gray as NCS pattern. We used two strains of zebrafish- AB/WT and Huc/GCaMP. Consistent with former research, larval zebrafish failed to associate white-black checkerboard with the US but learned pretty well with pure black CS pattern.

## Trial-by-trial analysis reveals different subtypes of learner and non-learners in larvae

* 1. Plot learning curve: learned, not learned, all (refer Jennifer Li.)
  2. Separate hist plot for learners and non-learners
  3. Self-abuse fish

After showed learning effect statistically, we want to learn more about individuals. To count the proportion of learners. Here, we divide the entire process into many trials, every 2 mins are counted as one trial. Therefore, the baseline has 5 trials, training has 10 trials and test has 9 trials. When the NCS area time proportion falls lower than mean NCS area time proportion level in the baseline, it is counted as the memory of fish extinct. When the PItime in the test is significantly higher than the PItime in the baseline.

Then we plot the learning curve along trials to see whether the learning actually happened.

Unexpectedly, we found 3 fish showed abnormal reverse-learning effect. (see fig.)

1. The relation between bias and learning effect

## Memory extinction in larval zebrafish

* 1. Fig.5 Show memory extinction histogram for each pattern-strain pair
  2. Fig.5 Plot memory extinction versus fish age

Memory extinction is known as an active learning process, when only CS presented without the US, subjects may learn the new association and do not show conditioned response again to CS. In this operant learning paradigm, the extinction time is defined as the time when mean NCS time proportion of the past 120 seconds first equals or lower than the value of the entire baseline. By using ABLITZER analysis toolkit, we found that the extinction time is positively correlated with learning effect.

## Ontogeny of operant learning in GCaMP for different conditioned pattern

* 1. Fig.4 Learning percentage changes with fish age (6dpf-10dpf)

## Calcium imaging of electric shock in naïve larval zebrafish

* 1. Set-up diagram
  2. Labeling
  3. Calcium changing curve

## Calcium imaging of abnormal fish receiving electric shocks

* 1. Experimental settings
  2. Labeling
  3. Calcium changing curve with visual stimulus

# **Discussion**

There might be some key genes of learning that can be screened out from the genetic difference between strain AB and strain Huc.

The set-up can easily be escalated to double the number fish in one experiment round.

Citation from Shuman’s paper: As this paradigm is simple and does not involve restraining of larvae, it can easily be serialized for high-throughput behavioral or pharmacological screens or proteomic approaches. Furthermore, as the learning difference between strains is significantly different, this paradigm may be ideally suited to screen out learning related key genes in zebrafish.

We provide a fully automatic end-to-end learning procedure for larval zebrafish and also two custom software for post data analysis, which can be easily adapted for running other associative learning paradigms in larval zebrafish. From scientific points of view, we demonstrated that 7-10 dpf WT/AB and Huc:h2b:GCamp fish both can learn the operant learning task and Huc fish learn better than AB fish in this task. Moreover, visual modality modulate operant learning effect in larval zebrafish, more preferenced the CS pattern is, better fish would learn.

In the future, we may develop a similar head-fixed or freely swimming paradigm to investigate the behavior-related neural calcium change online.

Just like the prevail of the computer is the first innovation of scientific labs. Automation would be the next generation.

Fish seems to behave much slower to recognize the red-black checkerboard than pure black, which might mean that larvae need more time to recognize or accumulate enough information to make the final decision.

# **Conclusions**

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

# **Author Contributions**

Wenbin

# **Appendices**

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

# **Declaration of interest**

This work was funded by ….

# **Acknowledgment (all references should be cited in the text and vice versa)**

This work was funded by ….

# **Reference (similar to Harvard citation)**

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