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A standardized protocol for preference testing to assess fish welfare

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Editorial Office

JoVE

15th November, 2019

Dear Editor,

We were most grateful for the constructive criticism of our manuscript (MS. No. JoVE60674), '*Assessing what zebrafish want: A test of enrichment and flow preference*', and for the opportunity to resubmit a revised version. We have edited the manuscript according to the suggestions from the reviewers.

Yours faithfully,

Cairsty DePasquale

Corresponding author

TITLE:

A Standardized Protocol for Preference Testing to Assess Fish Welfare

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KEYWORDS:

environmental enrichment, habitat preference, physical activity, swimming, welfare, zebrafish

SUMMARY:

A fundamental aspect of assessing the welfare of animals in captivity is to ask whether the animals have what they want. Here, we present a protocol to determine housing preference in the zebrafish (*Danio rerio*) with respect to the presence/absence of environmental enrichment and access to flowing of water.

ABSTRACT:

Animal welfare assessment techniques try to take into consideration the specific needs and wants of the animal in question. Providing enrichment (the addition of physical objects or conspecifics in the housing environment) is often a way to give captive animals the opportunity to choose who or what they interact with and how they spend their time. A fundamental component of the aquatic environment that is often overlooked in captivity, however, is the ability for the animal to choose to engage in physical exercise. For many animals, including fish, exercise is an important aspect of their life history, and is known to have many health benefits, including positive changes in the brain and behavior. Here we present a method for assessing habitat preferences in captive animals. The protocol could easily be adapted to look at a variety of environmental factors (e.g., gravel versus sand as a substrate, plastic plants versus live plants, low flow versus high flow of water) in different aquatic species, or for use with terrestrial species. Statistical assessment of preference is carried out using Jacob's preference index, which ranks the habitats from -1 (avoidance) to +1 (most preferred). With this information, it can be determined what the animal wants from a welfare perspective, including their preferred location.

INTRODUCTION:

The regulations governing how laboratory animals should be housed in captivity are explicit and well-defined. The Association for Assessment and Accreditation of Laboratory Animal Care

(AAALAC) International oversees and manages all organizations and institutions that work with research animals and has specific guidelines for species-appropriate husbandry and housing. For example, The AAALAC's *Guidance on the Housing and Care of Zebrafish, Danio Rerio*[1] "strongly encourages" the use of enrichment (the addition of physical objects or conspecifics in the housing environment) when housing zebrafish in captivity. The guide goes on to state, "Providing artificial plants or structures that imitate the zebrafish habitat allow animals a choice within their environment."

Evidence suggests that enrichment can stimulate the growth of new neurons (neurogenesis) in areas of the brain involved in processing spatial information[2], and it is thought that these neural changes are associated with enhanced learning ability[3]. The effects of enrichment on neurogenesis and learning have been widely studied across various taxa, including fish[4,5], birds[6], reptiles[7], and mammals[8]. Although these types of studies are important to understand the effects of enrichment on the brain and behavior, they do not take into consideration the particular choices or preferences of animals for a particular environment over another.

A fundamental question to ask when assessing the welfare of captive animals is whether or not the animals have what they want[9]. A way to investigate this question that provides tangible evidence is to provide animals with choices that allow us to understand their subjective preferences. For example, two studies have investigated whether zebrafish prefer access to either an enriched or a plain environment, with both studies indicating a preference for areas that contain enrichment[10,11]. However, it has also been suggested that zebrafish appear indifferent to environmental enrichment[12], so the answer to the question is obviously not clear-cut. Another application of preference testing associated with animal welfare extends to trying to understand how different aspects of an enriched environment play a part in the choices an individual animal makes. In fish alone, different types of enrichment have differential effects on the brain and behavior, and this relationship is further complicated by individual differences in personality traits[13]. Moreover, preference testing could be useful for comparative studies of environmental enrichment. Even across different fish species, enrichment has been shown to have an effect on many different types of behavior, including aggression[14], boldness[15], locomotion[16], and risk-taking behavior[17].

Jacob's preference index is a statistical test that is used frequently to quantify housing preferences[18]. Jacob's preference index assigns a value to each different habitat based on the number of animals present in each habitat type at different time points, where preference ranges from -1 (avoidance) to +1 (most preferred). Here we describe a method for using Jacob's preference index to investigate housing preferences in fish and use the example of assessing two important characteristics of the aquatic environment: 1) the presence or absence of enrichment; and 2) the flow of water[19]. However, the protocol could easily be adapted to look at a variety of environmental factors (e.g., gravel versus sand as a substrate, plastic plants versus live plants, low versus high water flow) across different species and landscapes (e.g., aquatic and terrestrial).

PROTOCOL:

The current study has approval and complies with all requirements of the animal care and use protocols of the Pennsylvania State University; IACUC no. 46466.

1. Setup of preference apparatus

1.1. Attain approval from the institute's Animal Care Committee (or equivalent organization) for all experimental and husbandry procedures involving live animals before commencing the experiment.

1.2. Use an experimental tank made of opaque white plastic. The walls between zones are made from grey acrylic that is fixed in place with silicon sealant.

NOTE: The size of the experimental tank is dependent on the size of the species of interest and the number of individuals used (e.g., for 8 adult zebrafish, a tank of 76 cm L x 76 cm W x 30 cm H is recommended).

1.3. Split the experimental tank into four zones that vary in accordance with the specific habitat parameters to be tested. Examples of different types of enrichment to investigate include sandy vs. rocky substrate, artificial plants vs. shelters, or flow of water vs. presence of artificial plants (**Figure 1**).

1.3.1. If using flow of water as a parameter of interest, use small pumps to supply jets of water (see **Table of Materials**). Set the pumps at a chosen velocity so that they provide a constant and directed flow of water. Choose the desired velocity based on the species of interest's ecology and life history (e.g., 14 cm/s for zebrafish).

1.4. In the middle of the experimental tank, have a central arena where food is delivered (**Figure 1**). Access to the central arena from each zone is through a small opening in the separating walls. The opening is large enough for the species of interest to move between zones unhindered, but small enough to reduce any visual cues the fish might experience from other zones.

1.5. Place a biofilter and a heater in each corner of the tank, but outside the experimental area so as not to disturb the flow of water and to ensure a constant water temperature across all zones.

1.6. Set up additional experimental tanks as space dictates. Rotate the different zones in each experimental tank to limit any sequential bias. Ensure that all replicate tanks have uniform conditions (same light levels, water temperature, etc.)

1.7. Place cameras (see **Table of Materials**) on tripods directly above each experimental tank, so that all zones are visible. Avoid wide-angle lenses and ensure the memory cards have enough space for recording.

1.8. Set the room lighting on a gradual (e.g., 1/2 h) 12 L: 12 D cycle to simulate sunrise and sunset.

Maintain water temperature at 25 ± 1 °C.

2. Capture, acclimation, and procedure

2.1. Keep fish in home tanks when they are not being tested. Net all test fish from their home tanks and place in the center arena of the experimental tank (Day 1). Minimize capture times to reduce stress (e.g., less than 30 s).

NOTE: An alternative procedure for transferring fish from their home tank to the experimental tank that may minimize stress is to transport the fish in a beaker of tank water.

2.2. Keep the number and gender of the fish in each experimental tank constant across replicate tanks and choose based on the species size and ecology.

2.3. On days 1–4, fish spend time acclimatizing and exploring the different zones. Do not collect data on these days.

NOTE: Extend or reduce the number of days for acclimation depending on the particular experimental protocol. However, the acclimation period should be sufficient to minimize the effects of handling as well as to get the fish accustomed to feeding in the apparatus.

2.4. During the acclimation period, monitor water quality closely by conducting regular water quality tests (e.g., pH, nitrate, or nitrite levels) and replace the water if any problems are detected (see **Table of Materials**).

2.5. Feed the fish flake food (see **Table of Materials**) in the central arena using a floating food ring (see **Table of Materials**) attached to the wall of the central arena at the water's surface. A food ring ensures food particles stay within the central arena and do not present a bias for zones due to food drifting.

2.6. Give the fish .5 h to feed ad libitum before removing the leftover food from the experimental tank with a dip net. Feed the fish once in the morning and once in the afternoon.

2.7. Assess behaviors on days 5–7. Switch cameras on and record fish behaviors for 2 h after each scheduled morning and afternoon feeding. On day 8 remove all fish from the experimental tanks with a dip net and place them back in their home tanks.

2.8. Depending on how much sump water is available, replace at least a 1/3 of the water in the experimental tank with fresh sump water to reduce any effects of stress hormones on fish in following replicates.

2.9. Set up the experimental tanks in accordance with the zone rotation schedule for that week. Rotating the zones decreases the chance of any behavioral bias occurring as a result of the placement of any zone relative to each other. Then begin the testing process again with a new

batch of fish.

3. Measurements and data analysis

3.1. Download the videos to a computer at the end of each recording day. This ensures there is space on the memory card before every use.

3.2. Use video software (see **Table of Materials**) to quantify zone preference. Manually count the number of fish in each zone at 5 min intervals in each 2 h recording period (include the central arena in these counts). Define the gender of the fish during analysis if differentiation between males and females is possible from the video footage.

3.3. To analyze habitat preference, calculate the mean number of fish per zone for each replicate tank (i.e., average all data across the 3 days). In order to obtain a preference score for structure use, calculate Jacobs' preference index[15] as

$$J = (r_x - p)/[(r_x + p) - 2*r_x*p]$$

where x is the zone of interest, r_x is the ratio of fish in zone x to the total number of fish in all zones, and p is the available proportion of all zones in the experimental tank. The index ranges between +1 for maximum preference, and −1 for maximum avoidance.

3.4. To determine if there are any changes in the rate at which fish switch between zones during an observation period, calculate the switch rate, r_{sr}, in the first and last 5 min of every observation period, where r_{sr} is the number of times a fish enters each zone from the central arena, divided by the total number of fish.

3.5. Consider a fish to have entered into a zone when the fish's whole body crosses through the opening separating the zones. Calculate a starting and a finishing mean switch rate for each replicate tank. Carry out all behavioral observations by the same experimenter to reduce any experimenter observation bias.

3.6. Using statistical software (see **Table of Materials**), conduct relevant statistical analyses. Suggested analyses include a one-way ANOVA, with preference index as the dependent variable and zone as the predictor variable, and a paired t-test on the starting and finishing mean switch rate for each tank.

3.7. Apply Tukey's multiple comparison post hoc test to further investigate zone comparisons, where each zone is compared to each other. More complex statistical analysis includes mixed models that assess time effects, arena effects, sex effects, or even individual differences in behavior.

REPRESENTATIVE RESULTS:

We used the preference test to investigate housing preferences in zebrafish given a choice

between varying enrichment including 1) plastic plants and sandy substrate; and 2) water flow. These were divided into four zones: (i) Enriched Only; (ii) Flow Only; (iii) Enriched and Flow; (iv) Plain; and a Central arena where food was delivered[19]. Zebrafish showed the highest preference for the Enriched and Flow zone, which was significantly different than all other zones (Enriched Only, Flow Only, Plain, and Central Arena; $p < 0.01$). Fish avoided both the Flow Only and Plain zones, spending more time in the Central Arena[19] (**Figure 2A**). In addition, zebrafish moved between different habitat zones more often at the start of the observation period than at the end (**Figure 2B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Examples of different experimental designs to test for habitat preferences. (**A**) Setup of an experimental tank to test the preference of a sandy versus a rocky substrate. (**B**) Setup of an experimental tank to test the preference of enrichment (plastic plants) versus a shelter. (**C**) Setup of an experimental tank to test the preference of enrichment (plastic plants) versus a flow of water. In all figure panels, the four corner compartments were not accessible to the fish and only contained heaters and filters.

Figure 2: Representative data showing the results of a habitat preference test on zebrafish. (**A**) Jacobs' preference index for each zone: (**i**) Enriched only; (**ii**) Enriched and Flow; (**iii**) Flow Only; (**iv**) Plain; and a neutral Central Arena. Positive and negative values indicate preference and avoidance, respectively. The boxes indicate the 25 ± 75th percentile range and contain the median line; bars represent the 10th and 90th percentile values; open dots represent points outside these values. a = significant difference from all zones ($p < 0.05$); b = significantly different from Enriched and Flow, Enriched Only, and the Central Arena ($p < 0.05$); and (**B**) box plots showing the switch rate at the beginning and the end of the observation period (boxes indicate the 25 ± 75th percentile range and contain the median line; bars represent the 10th and 90th percentile values). **Figure 2A** has been modified from DePasquale et al.[19].

DISCUSSION:

Here we present an experimental design that allows us to investigate the preferences of fish for different types of habitats. Some critical steps that are important in preference testing include: 1) ensuring that uniform conditions are maintained across different replicates (e.g., external noises or movement, experimenter, water chemistry, light levels); 2) ensuring that the zones are rotated between replicates and a significant amount of water is replaced with fresh sump water between tests to reduce biases; (3) ensuring that an appropriate sample size is used to detect significant results, both in terms of number of individuals in each group and number of replicate tanks; and 4) if trials are recorded, optimizing and ensuring proper video recording and file transfer.

Modifications to the current protocol include exposing fish to a variety of other habitat types, such as different enrichment items, different substrates, or even different flow rates. In addition, it may be possible to use animal tracking software to further understand how the fish are using the space in each zone (e.g., do the fish spend time swimming against the flow of water in the flow zones, or do they avoid that part of the habitat altogether). However, the walls of the

experimental tank may need to be modified to accommodate this type of tracking software. Finally, the preference test described here could be adapted to any fish species, or potentially any aquatic organism that the experimenter wants to investigate.

A limitation of the current protocol is that preference testing is limited by the resources that are presented to the animals. Therefore, the animal may not be choosing a preferred choice, but the least unpleasant of those presented[20]. However, it may be that having a choice in the first place is better for welfare than only being given limited options (i.e., access to the most preferred habitat only). Also, it has been suggested that zebrafish find light backgrounds aversive[23], thus an alternative tank color (e.g., black) may be more suitable. Moreover, preference testing is often limited to observations made in a small window of time, where the animal in question may be acting on immediate cues rather than future needs[21,22]. In addition, gender, group size, and social context are factors that affect group dynamics and therefore potentially habitat preferences in fish, so it is important to try to keep these factors consistent across replicates.

With our representative results we showed that zebrafish preferentially choose both Enriched and Flow and Enriched Only zones and avoid Flow Only and Plain Zones. In sum, the Enriched and Flow zone was preferred over all other zones. A preference for enriched environments, and in particular the Enriched and Flow Zone, may be the result of an increased need for sensory stimulation (exploration) or it could be the need to find places to hide (reduced competition from conspecifics). Interestingly, there was a slight preference for the Central Arena over the Flow Only and Plain zones, suggesting that the potential of food being delivered was a higher motivational factor than swimming. In terms of movement between the zones, there was more switching between zones in the beginning of the observation period than at the end. The increase in movement at the beginning of the observation period may correspond to the timing of feeding (fish were fed half an hour before recording started), thus they may have been more motivated to move and look for additional food. In summary, the protocol described in the current study is an effective tool for looking at habitat preferences in fish.

ACKNOWLEDGMENTS:
This work was supported by a Research Collaboration Fellowship and the Huck Institute at The Pennsylvania State University, as well as USDA AES 4558. The research complied with all requirements of the animal care and use protocols of the Pennsylvania State University; IACUC no. 46466.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

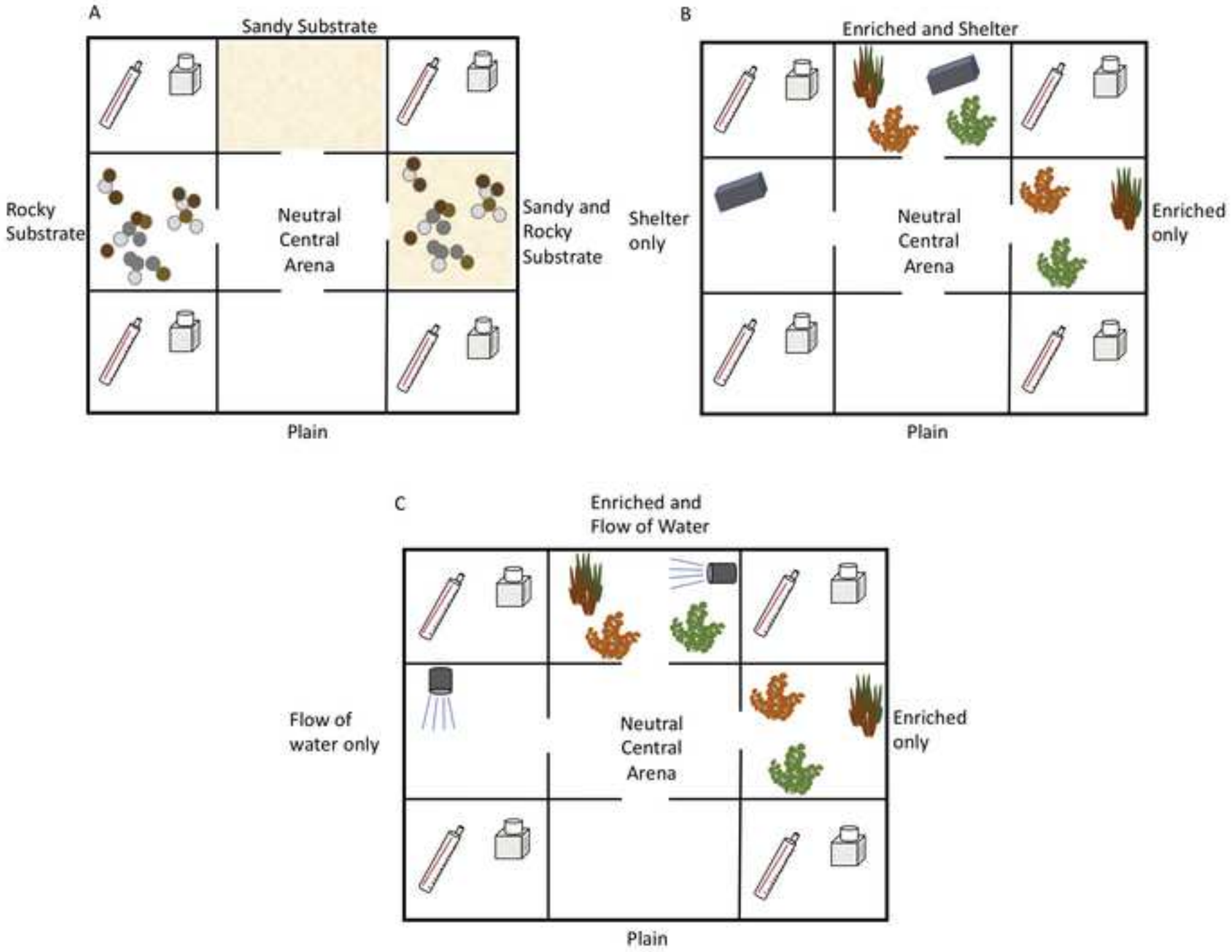

Figure 2

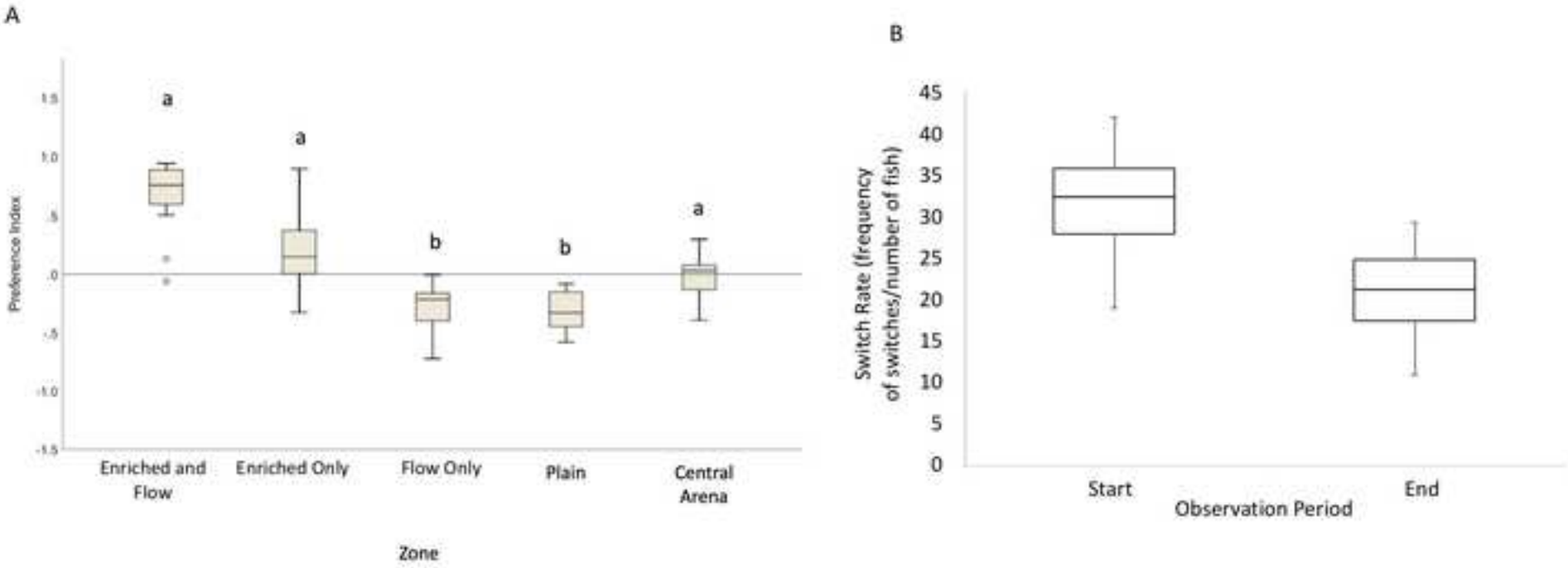

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_Depasquale et al.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Artificial Aquarium Plants	Smarlin	B07PDZQ5M5	
Artificial Seaweed Water Plants for Aquarium	MyLifeUNIT	PT16L212	
Experimental tanks	United State Plastic Corporation	6106	
Floating food ring	SunGrow	B07M6VWH9V	
Flow meter	YSI	BA1100	
Jager Aquarium Thermostat Heater	Ehiem	3619090	
Master Water Quality Test Kit	API	34	
SPSS Statistics for Macintosh	IBM	Version 25.0	
Submersible Pump, SL-	Songlong	SL-381	
TetraMin Tropical Flakes	Tetra	16106	
Triple Flow Corner Biofilter	Lee's	13405	
Video camera	Coleman	TrekHD CVW16HD	
Windows Media Player (video software)	Microsoft	Windows Media Player 12	

Response to Referees:

We are very grateful to both the reviewers and the Editor for their constructive feedback and suggestions. We have now thoroughly revised the manuscript according to the suggestions and we believe that this revision process has helped clarify and strengthen the manuscript. Below we highlight the changes that have been made and our replies to the specific questions.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
- Done
2. Please rewrite the title to avoid the use of a subtitle.
- Done
3. Please revise lines 49-54, 59-66, 72-78, 105-107, 126-135, and 139-162 to avoid textual overlap with previous publications.
- The introduction has been thoroughly revised and re-written to exclude any overlap with previous publications, as well as to attract a more general audience.
4. Please provide email addresses for all authors in the manuscript.
- Added, with the exception of Victoria Braithwaite

Protocol:

1. Please ensure that each step/substep contains mainly instructions in the imperative.
- Done
2. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
- Done

Specific Protocol steps:

1. 2.1: Please move this statement to the beginning of the protocol section, and also indicate that this procedure has been approved by your own institution’s committee.
- Completed. See lines 88-91
2. 3: Much of this section consists of vague calculations that cannot be filmed.
- This section has been re-worded (see lines 126-130)

Figures:

1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

- I have attached email correspondence with one of the Editors of the journal (AABS). She said in an email dated November 8th to go ahead with this submission and granted permission to use figure. If you require additional permission, please let me know.

2. Please combine Figure 2A and 2B into one image file.
- Done

References:
1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source.* **Volume** (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
- Corrected
2. Please do not abbreviate journal titles.
- Corrected

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
- Added

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The reviewed paper is a description of a methodology to assess the environmental preference of fish (zebrafish in particular). I believe that a video-paper on this could be very interesting. However, I think that the paper should be written in a more general way, so that it attracts more users (applying it on different species, and using different environmental factors).

Major Concerns:
There are no major concerns regarding the validity of the protocol.

Minor Concerns:
Title:
For a paper describing methodology, it might be better to use a more general title. The test of whether zebrafish prefer enrichment or flow specifically, is already done (DePasquale et al. Appl. Anim. Behav. Sci), and the methodology should be well described in that paper already. I think that the experimental setup is generalizable to many different environmental factors, and scalable to be used for many different species. Sure, it may be possible to test e.g. different strains in the exact same setup, but it would be more interesting to test different species and different environmental factors.
- Title changed to something more general

Also, is it really a test of "flow"? To me, it seems to be a test of the presence of turbulence… "Flow" would be unidirectional in my mind.
The definition of flow that we are using is, ‘a fluid moving along continuously in a current or stream’. This definition does not define whether there was turbulence or not. Thus, we feel the word ‘flow’ will suffice.

I would suggest a title like: "Assessing what fish wants: a standardised preference test setup"
Then, present the enrichment x turbulence setup for zebrafish as an example (and skip the predictions of the treatments from the abstract). This would likely attract more users and citations.
- Title changed.
- Specific treatment groups used in DePasquale et al. Appl. Anim. Behav. Sci. taken out of abstract to make the context of the preference test more general.

Introduction:
I suggest widening the scope a bit, to make the paper less overlapping with the already published study. Focus more on the test itself, and the usefullness of preference tests in the context of fish welfare, and less on zebrafish, enrichment and exercise specifically. It would be useful if the authors could expand their suggestions of other environmental factors to test, and emphasize the possibility of running the test for fishes in general. For instance, it could be a useful test for comparative studies in environmental preference. Different species have different responses to e.g. enrichment. Furthermore, different types of enrichment could be compared in this setup, since that also seems to affect the results obtained in enrichment studies (see. e.g. Näslund & Johnsson 2016 Fish and Fisheries)
- The introduction has been written to reflect a larger focus on the usefulness of the preference test for assessing fish welfare in general, rather than the specific application of the test from DePasquale et al. Appl. Anim. Behav. Sci.

Line 49: Revise to: "Evidence suggests that enrichment can stimulate neurogenesis…" or similar. Effects seem to differ across different species and experiments. Also, the evidence in the studies indicating positive effects is probabilistis - so "is known to" is still a very bold statement…
-Revised see lines 52-53

Protocol:
Point 1.3: Applying both sand and a variety of different shelters and artificial plants makes it impossible to figure out which of these factors affects the preference for the enriched chamber. Mixed enrichment treatments are not recommended for this reason. This part of the protocol could be more generalized.
- Revised to include more general instructions on the habitat zones (see lines 96-103)
Point 1.4 & 1.5: These points are very specific for "flow" (turbulence?) treatments. Again, I would suggest a more generalized protocol. The description of the flow

environment could be put under a more generalized Point 1.3 (e.g. as 1.3b, with 1.3a being a description of the enriched environment).
- - Revised to include more general instructions on the habitat zones (see lines 96-103)
Point 1.7: Why should there be specifically three tanks per week? If I have the space for more tanks, wouldn't it be better to adjust the number of tanks to a larger number?
- Revised to say, 'Set up additional experimental tanks as space dictates.'
Point 1.8: Some details on the camera equipment and settings (e.g. avoiding wide-angle lenses) would be useful.
- Added. See lines 114-115.
Point 2.2: Could be more general. It is surely possible to use any number of fish and sex-ratio. The specific 4+4 protocol is only necessary if the experimenter wants to replicate the particular zebrafish study that the authors have published.
- Taken out specific number of fish and kept instructions more general (see lines 124-125)
Point 2.3: I think this way of presenting the protocol is better, since it is more general (acclimation time adapted to the experimenter's own protocol).
- Noted
Following my previous points, I think that all points should be more general - so that it relates to a wide range of species and environmental factors applied in the preference chambers.
- The protocol has been thoroughly revised to make it more general to different species and environmental factors (e.g. Figure 1)
Point 3.5 & 3.6: Analyses should probably be "Suggested analyses". It would be possible to run more efficient models to test these things (e.g. mixed models for assessing time effects and arena effects of differences in start vs. finishing switch rate - this may be overkill, but I think there can be reasons to apply more complex models sometimes).
- Changed (see lines 172-179)

Representative results: This section is fine, I think.

Discussion:
Line 188: The point about uniform conditions should be made in the protocol (and re-stated in the Discussion). Also provide some information about how to measure and ensure environmental homogeneity (add the necessary equipment to the equipment table).
- Added in protocol (see lines 131-134). Also added testing equipment to materials table

Line 196: The walls of the experimental tank will make it hard to do tracking, since the fish could be hidden behind the walls (obvious from the photograph of the setup) - but it may be possible with certain softwares, or with modified arenas…?
- Edited to add, 'however, the walls of the experimental tank may need to be modified to accommodate this type of tracking software.'

Other comments:
Please include information about which software was used to score the preference.

-Added (see step 3.2 in protocol and the table of materials)

The photograph needs to be of higher quality. Maybe the figure has been added to the PDF in a lower resolution, but if not, one needs to be able to read the lables that are put on top of the arena walls. As is, I cannot read these labels.
-The photo used for Figure 1 has been replaced with a schematic diagram that gives a more general representation of how the experiment could be set up.

Please provide data points to figure 2b. Alternatively, use boxplots as in 2a. As a reader, and potential user of the test, I would like to see the actual data distribution to assess the relevance of the differences in switch rate.
- Figure 2b has been converted to boxplots.

Reviewer #2:
Manuscript Summary:
The MS provides a very useful and clear overview of how to implement a free-choice preference experiment in fish. I think the work will make a valuable contribution to the field and only have one major comment and several minor ones.

Major Concerns:
Though the authors acknowledge difficulties in assessing motivation to improve animal welfare (e.g., line 62 and line 202), I do not think they provide sufficient qualification to the limitations of the proposed methodology and motivation tests in general. For example, if we used Jacob's preference index (or any time-based preference test) to evaluate the importance of bathrooms to humans, we would probably discover that humans avoid bathrooms, yet it would be wrong to conclude that bathrooms should be removed to improve human welfare. Similarly (and not a particular fault of Jacob's preference index, but rather of motivational tests in general), consideration needs to be given to the value of having choices at all—having the opportunity to choose *between* preferred and dis-preferred options may be better for welfare than only having the preferred option. Some recent literature on the role of motivation in animal welfare may be instructive and should be discussed:
* Fraser & Nicol (2018). Preference and motivation research. In: Animal Welfare, Third Edition pp 213-231. CABI
* Gygax & Hillmann (2018). "Naturalness" and Its Relation to Animal Welfare from an Ethological Perspective. Agriculture
* Franks (2019). "What do animals want?" Animal Welfare
- Thank you for bringing these references to my attention. I have added a section in the discussion to reflect some of the points brought up here (see lines 231-234)

Minor Concerns:
a) I do not think the size of the choice tank is specified, but at any rate, it should be discussed in relation to the size and group sizes of the study species.
- Added to protocol as a note (see lines 94-95)
b) 1.7 Why keep fish in standard housing conditions and what is meant by standard? How standard (perhaps a more neutral word like home-tank is better?) is defined will

interact with preferences (at least because of familiarity biases) and that factor should also be discussed/addressed.
- Changed to home tanks
c) 2.2 Specify target ranges for minimizing capture time and note that netting and removing from water is not advised as it is extremely stressful to the fish—alternative procedures would be preferred (eg capture and transport in water) and should be noted.
- Added (see lines 120-123)
d) 3.2 r is defined, but rx is not. Is this a typo?
- changed to 'where x is the zone of interest, r is the ratio of fish in zone x to the total number of fish'

e) Sometimes number of fish in experiment is specified as 8, sometimes it is referred to generically. Perhaps better to leave as generic throughout?
- Number of fish not specified to make procedure more general
f) 3.5 The recommended statistics are a bit limited and would require a large numbers of animals to have good power. Perhaps allow for other, more sophisticated methods that can investigate preferences over time and thus reduce the need for large replicates of animal groups.
-Added (see lines 172-179)

g) Perhaps some discussion/consideration is needed regarding sample size—both in terms of minimum group size required for this methodology and minimum number of groups needed to power the detection of different preference differentials.
- Added (see lines 234-236)
h) The test tank is white, which as been suggested to be aversive to zebrafish. Can the authors comment on the trade-offs of using different background colors?
- Added (see lines 237-238)

Reviewer #3:
Manuscript Summary:
The manuscript discusses an important topic for facility managers, scientists, and technicians who work with fish models in laboratory research and who want to improve the welfare of laboratory-maintained fish. It will also be of interest to fish biologists and those involved in aquaculture and the ornamental fish industry. It is generally accepted that environmental enrichment improves the welfare of captive animals, including fish, and that better welfare leads to better science in research. The protocol described in the manuscript investigates the preferences of zebrafish and for water flow and for environmental enrichment in the form of structures added to the tank. Providing fish with choices rather an imposing enrichment upon them is vital to understanding their subjective preferences and binary choice tests are widely used in animal welfare research to make recommendations regarding the husbandry of captive animals. Although the manuscript describes a useful protocol, I recommend that it be revised as per the comments listed below.

Minor Concerns:

Lines 23-27: I suggest deleting the following sentences: "A fundamental component of the captive environment that is often overlooked, however, is the ability for the animal to choose to engage in physical exercise. For many animals, including fish, exercise is an important aspect of their life history, and is known to have many health benefits, including positive changes in the brain and behavior." The paper describes a protocol to measure habitat preference in fish, it does not measure aspects of physical exercise.
-This has been removed, and the abstract has been re-written from a more general perspective

Lines 32 and 80: I suggest removing the word 'main' as it implies that more than one measurement of preference was used whereas the manuscript describes only one.
- Removed

Lines 49-52: The two papers cited in lines 49-52 discuss neurogenesis in animals in general (ref. 2) and in humans (ref. 3) but not specifically in zebrafish. The sentence which begins "For example, zebrafish reared with enrichment…" implies that the previous citations refer to zebrafish. Deleting "For example" would fix this.
- This paragraph has been edited and those citations have been moved to another part of the introduction (see lines 52 to 74)

Line 92: It would be useful to include information about tank dimensions.
- Added (line 94)

Lines 98-99: The protocol states that sandy substrate and shelters should be used as enrichment items but these are not visible on Figure 1.
-Figure 1 has been edited to include various experimental set ups to make it more general across different species and types of enrichment

Line 98: Why should a sand substrate be provided when zebrafish are known to prefer gravel?
- This part of the protocol has been edited to more generalized descriptions of what could be used as substrate or enrichment items (see lines 96 to 103)

Line 101: I suggest that the actual velocity used for the zebrafish experiment be stated, followed by a note that this velocity may be changed depending on the species being tested.
- Changed (see lines 101 to 103)

Line 108: The justification for using 3 replicates per week and 8 fish per tank should be added, perhaps in the Introduction.
- Changed to, ‘Set up additional experimental tanks as space dictates’ to keep the instructions more general

Line 112: How long was the L:D transition phase?
-Information added (see line 116)

Line 113: Was water quality monitored? As each test lasted for 7 days and only one third of the water was changed between replicates, water quality likely deteriorated over the course of the experiment.
- Yes, water quality was checked. Detalis about this was added (see lines 131-133)

Line 134: Add a brief explanation as to why a third, rather than all, of the tank water should be changed between replicates.
-Added, see lines 143-145

Lines 164-165: According to data on Figure 2A, zebrafish show a preference for the Enriched+Swimming and the Enriched only zones…
- Results reworded (see lines 181-188)

Lines 208-211: I suggest adding a brief explanation for the representative results. Why might zebrafish prefer the Enriched+Swimming and Enriched only zones but avoid the Swimming only and Plain zones? Why might zebrafish move between the habitats more often at the start of the observation period than at the end?
- Added (see lines 242-251)

Line 209: Add 'and the Enriched only zone' after 'Enriched+Swimming zone'.
- Added

Table of Materials: The tank, enrichment objects, water pumps, biofilters, heaters, and floating food ring should be added to the Table of Materials.
- Added

Figure 1: The labels in Figure 1 do not match the zone names in lines 92-93.
-The photo used for Figure 1 has been replaced with a schematic diagram that gives a better representation of how the experiment could be set up.

From: **Sylvie Cloutier** SCloutier@CCAC.CA
Subject: Re: Request for copyright permission
Date: November 13, 2019 at 8:49 PM
To: Depasquale, Cairsty ocg102@psu.edu

SC

I will be back to my office tomorrow and will take care of it. We just found out that our publisher manager is out on sick leave. So I will take the responsibility for this. I am traveling (actually on the train as I write) back home from a business trip and will get that done tomorrow. Sorry for all the stress this has caused.

Best wishes,
Sylvie

From: Depasquale, Cairsty <ocg102@psu.edu>
Sent: November 13, 2019 8:24 PM
To: Sylvie Cloutier
Subject: Re: Request for copyright permission

Hello Sylvie,

I have contacted Elsevier's research support team about this matter, to no avail. I am sorry to have to email you again about this, but it would be really helpful if you could sign the letter providing copyright permission and send it back to me. Unfortunately, I do not think our email correspondence will suffice as permission, and it needs to be in the form of a letter that can be uploaded with the manuscript. The deadline for me to upload the letter is friday 15th.

Thanks for your assistance.

Cairsty

Cairsty DePasquale, Ph.D.
Associate Professor of Biology

207 Hawthorn Building
3000 Ivyside Park
Penn State Altoona
Altoona, PA 16601
Office: (814) 949-5287
Website: www.personal.psu.edu/ocg102/

> On Nov 11, 2019, at 1:55 PM, Depasquale, Cairsty <ocg102@psu.edu> wrote:
>
> Hello Sylvie,
>
> To make things easier, I have attached a letter that you can use to sign and send back to me. Let me know if there are any issues with the letter and I can make edits as needed.
>
> Thank you for your assistance in this matter.
>
> Best wishes,
>
> Cairsty
>
> > On Nov 8, 2019, at 11:53 AM, Depasquale, Cairsty <ocg102@psu.edu> wrote:
> >
> > Thank you, Sylvie.
> > Would it be possible to acquire a formal letter stating this that I can upload with my manuscript?
> >
> > Best wishes,
> >
> > Cairsty
> >
> > Dr. Cairsty DePasquale
> > Associate Professor of Biology
> >
> > 207 Hawthorn
> > 3000 Ivyside Dr.
> > Penn State Altoona
> > Altoona, PA 16601
> > Office: 814-949-5287
> > Website: www.personal.psu.edu/ocg102/
> >
> > > On Nov 8, 2019, at 11:47 AM, Sylvie Cloutier <SCloutier@CCAC.CA> wrote:
> > >
> > > Dear Cairsty,
> > >
> > > Just go ahead. I did not get any reply from the journal manager and publisher.
> > >
> > > Best wishes,
> > > Sylvie
> > >
> > > **Sylvie Cloutier, PhD**
> > > Directrice adjointe d'évaluation I Associate Director of Assessment
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From: Depasquale, Cairsty [mailto:ocg102@psu.edu]
Sent: November-08-19 11:44 AM
To: Sylvie Cloutier
Subject: Re: Request for copyright permission

Dear Sylvie,

Is there an update on my query?
Is there anything I can do to help find the answer? I am concerned that I will not be able to submit the revisions to my manuscript on time (the deadline is the 15th November).

Any help you can give me is much appreciated.

Cairsty

Dr. Cairsty DePasquale
Associate Professor of Biology

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Altoona, PA 16601
Office: 814-949-5287
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On Oct 7, 2019, at 12:17 PM, Sylvie Cloutier <SCloutier@CCAC.CA> wrote:

Dear Dr. Depasquale,

This is a first for me since I have been an editor for AABS. So, I am checking with the publisher about who is responsible for allowing this type of permission. I am hoping we can get back to you shortly.

Best regards,
Sylvie

Sylvie Cloutier, PhD
Directrice adjointe d'évaluation I Associate Director of Assessment
Conseil canadien de protection des animaux I Canadian Council on Animal Care

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From: Depasquale, Cairsty [mailto:ocg102@psu.edu]
Sent: October-03-19 11:02 AM
To: eesserver@eesmail.elsevier.com; Sylvie Cloutier
Subject: Request for copyright permission

Dear Editor,

Please find attached a letter requesting copyright permission for two figures in the attached publication from Applied Animal Behaviour Science. I would appreciate your response to my request. If you have any questions, please do not hesitate to contact me.

Sincerely,

Cairsty DePasquale

Cairsty DePasquale, Ph.D.
Associate Professor of Biology

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<Letter to AABS Editors_Signature Form.docx>