Video Article

Boldness, Aggression, and Shoaling Assays for Zebrafish Behavioral Syndromes

Gregory P. Way^{1,2}, Maura Southwell¹, Scott P. McRobert¹

¹Department of Biology, Saint Joseph's University

²Genomics and Computational Biology, University of Pennsylvania

Correspondence to: Scott P. McRobert at smcrober@sju.edu

URL: <http://www.jove.com/video/54049>

DOI: [doi:10.3791/54049](https://doi.org/10.3791/54049)

Keywords: Medicine, Issue 114, Zebrafish, behavior, behavioral syndrome, personality, boldness, aggression, shoaling

Date Published: 8/29/2016

Citation: Way, G.P., Southwell, M., McRobert, S.P. Boldness, Aggression, and Shoaling Assays for Zebrafish Behavioral Syndromes. *J. Vis. Exp.* (114), e54049, doi:10.3791/54049 (2016).

Abstract

A behavioral syndrome exists when specific behaviors interact under different contexts. Zebrafish have been test subjects in recent studies and it is important to standardize protocols to ensure proper analyses and interpretations. In our previous studies, we have measured boldness by monitoring a series of behaviors (time near surface, latency in transitions, number of transitions, and darts) in a 1.5 L trapezoidal tank. Likewise, we quantified aggression by observing bites, lateral displays, darts, and time near an inclined mirror in a rectangular 19 L tank. By dividing a 76 L tank into thirds, we also examined shoaling preferences. The shoaling assay is a highly customizable assay and can be tailored for specific hypotheses. However, protocols for this assay also must be standardized, yet flexible enough for customization. In previous studies, end chambers were either empty, contained 5 or 10 zebrafish, or 5 pearl danios (*D. albolineatus*). In the following manuscript, we present a detailed protocol and representative data that accompany successful applications of the protocol, which will allow for replication of behavioral syndrome experiments.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54049/>

Introduction

There is a growing body of literature investigating the associations between distinct behaviors within individual animals from a given population. These associations are termed behavioral syndromes, and the measurements typically include boldness, aggression, exploratory behavior, and sociability¹⁻⁵. Behavioral syndromes are valuable for both direct and indirect reasons. Directly, knowledge of behavioral syndromes can provide a more complete view of evolutionary theory, population structure, and population dynamics³. Indirectly, knowledge regarding behavioral associations may inform fields that quantify behavior such as pharmacology⁶, toxicology⁷, behavioral genetics^{8,9}, and endocrinology¹⁰. Because of these direct and indirect benefits, an increased knowledge of behavioral syndromes is especially valuable in commonly used model organisms such as the zebrafish. Studies using zebrafish are found in a variety of disciplines, including the analysis of behavioral syndromes¹¹⁻¹³. To advance knowledge in behavioral syndrome research, and because other disciplines also use behavioral measurements for hypothesis testing, reliable and succinct descriptions of behavior are required for valid analyses and interpretations and standardized protocols will facilitate inter-study comparisons within species. Our protocol was developed to measure a boldness-aggression-shoaling behavioral syndrome in a population of lab-reared zebrafish¹⁴. However, the basis of the protocol (tracking individual fish, ensuring proper randomization, and appropriate analyses) can be easily modified for a variety of alternative behavioral measures. Additionally, boldness, aggression, or shoaling assays can be run individually for the testing of distinct hypotheses. Therefore, while it is our goal to describe how to conduct a behavioral syndrome study and the protocol for successful individual level behavioral measurement, each facet of this procedure can stand alone.

The literature on behavioral syndromes spans several taxonomic groups, from arthropods to humans⁴ and, in order to measure a behavioral syndrome, at least two behavioral contexts must be quantified. Unfortunately, there is often little consistency in the assays that are used to quantify the behavioral measurement across the axes of behavior. For example, in fish, boldness may be measured using T-maze assays, open-field assays, or introduction of a novel or foreign stimulus¹⁵. Aggression studies in fish might involve dyad interactions, video stimulus assays, or clay model assays^{12,16,17}. Likewise, analysis of shoaling behavior, which typically involves the measurement of shoalmate preference, may be performed in different types of tanks, with different methods to determine association time²¹⁻²³. In this protocol a specific subset of the overall behavioral assay repertoire is presented. Specifically, this protocol presents a methodology to track individuals through boldness, aggression, and shoaling assays in a way that facilitates comparisons within individuals to determine whether the comparisons are consistent across all individuals within a population. We have performed this protocol with zebrafish and convict cichlids (*Amatitlania nigrofasciata*) in previous studies^{14,18}, and it will work with any similarly sized freshwater fish.

Boldness assays are conducted in a 1.5 L trapezoidal tank that has a horizontal line delineating equal sized areas in the upper and lower portions of the tank. Quantified behaviors include the number of transitions by the test fish between the upper and lower portion of the tank, the time spent in each portion, the number of darts, and the latency to enter the upper portion. The aggression assay is performed in a 19L

rectangular tank that includes a 3 inch x 5 inch mirror inclined at about 22° situated in the lower left corner of the tank¹⁹. Quantified behaviors include the total amount of time spent by the target fish interacting with the mirror²⁰, along with specific aggressive indicators – number of bites, lateral displays, darts between the test fish and its reflection. For these specific indicators, bites are defined as quick lunges toward the mirror with an open mouth, lateral displays are defined as the flaring of lateral, pectoral, anal and dorsal fins in the direction of the mirror, and darts are any erratic movements that are not directed toward the mirror. Lastly, the shoaling assay quantifies the behavior of a test fish in center chamber of a tri-chambered tank. The side chambers of the tank are either empty, or contain a "target shoal" of fish, and the time the test fish spends near each side chamber is measured²¹⁻²³. A single composite score, referred to as Strength of Shoaling (SoS), is calculated for each individual test fish, specific to the stimuli, and can be used in downstream analyses¹⁴. All behaviors are scored by a single viewer, or multiple viewers using free behavioral quantification software known as JWatcher²⁴.

Testing the presence of a behavioral syndrome is primarily a statistical endeavor, and it is advisable to follow the guidelines as presented by Budeav 2010²⁵. Specifically, it is recommended to perform a principal components analysis (PCA) on centered and normed data in which the inputs are the vectors of an individual's behaviors in assays with multiple behavioral measurements (*i.e.*, boldness and aggression). The PCA, performed on a correlation matrix, reduces the dimensionality of the behavioral measurements, and thus extracts the most important knowledge that explains a majority of the variation. The extracted components can then be interpreted based on high factor loadings for the individual behavior of interest and regression scores can be extracted for each individual on the basis of the explanatory components. These regression scores can then be compared to the SoS measurement and other various non-behavioral measurements such as fish size or sex.

This workflow has been implemented in a study of zebrafish behavioral syndromes in which a sex specific behavioral syndrome that exists between boldness and shoaling¹⁴ was discovered. In this situation, bolder zebrafish males are more likely to associate with a larger, more aggressive species (*D. albolineatus*), but this association is lost in females. This workflow was also implemented in a study of juvenile convict cichlid kin (*Amatitlania nigrofasciata*)¹⁸ in which a behavioral syndrome was not discovered, potentially indicating behavioral plasticity of the species. Therefore, the following protocol is presented with a goal of delineating the nature of three specific assays (boldness, aggression, and shoaling) in the framework of studying an individual level behavioral syndrome.

Protocol

The following methodologies for the housing, care, and study of zebrafish have been approved by the Saint Joseph's University IACUC.

1. Zebrafish Housing and Care

1. Obtain the subject zebrafish from a local supplier, or from wild-caught populations. Please note that housing the zebrafish is subject to IACUC guidelines and additional permits are required for housing wild-caught populations.
2. Identify and separate zebrafish according to sex. Alternatively, skip this step or replace with a different method of separation based on the study hypothesis.
NOTE: Sex characteristics are difficult to determine in zebrafish, particularly in females. Refer to these guidelines¹⁰ or useful online manuals to distinguish sex.
3. Randomly separate the populations further into the appropriate ratio of focal fish to stimulus fish. Note: Focal fish are the test subjects followed through the series of behavioral assays, and stimulus fish are the individuals that will occupy the stimulus chambers in the aggression and shoaling assays.
NOTE: Traditionally, between 1 and 5 stimulus fish per focal fish in shoaling assays and a 1:1 stimulus:focal fish ratio in aggression assays^{14,17} are used. However, the researcher needs to specifically design the study with the hypothesis in mind (*i.e.*, should stimulus fish be kept constant to maintain consistency in focal fish behavior, or should stimulus fish be rotated between each assay to ensure proper randomization).
4. Store all fish in zebrafish housing racks (27.5-28.5 °C) under a strict 12:12 light/dark cycle. Feed all focal and stimulus fish flake food daily and brine shrimp weekly, on non-research days.
5. Be sure that all testing is done between ZT1 and ZT5 (zeitgeber time) to avoid circadian rhythm effects²⁶.

2. Randomization and Tank Setup

1. Randomize all individuals *a priori* to a specific sequence of behavioral assays. Set up a consistent randomization scheme using an online random sequence generator²⁷ or a random sequence generator from a statistical software package (R).
 1. Label each assay from 1 to 3 and keep this assignment consistent. Assign a random sequence to each individual and map the sequence according to the behavioral assay label. For example, for three total assays (1 mapped to Shoaling, 2 mapped to Boldness, and 3 mapped to Aggression), a given individual may have a randomized sequence of '2,3,1', which will require them to perform the specific behavioral assays in the specified sequence.
2. Set up the assay tanks (boldness, aggression, and shoaling) in the laboratory in places that minimize the possibility for adverse external stimuli and be sure to have enough room for a video camcorder to capture the entire tank. Ensure that the camcorder has enough clarity to view tank labels as well as be able to distinguish subtle behaviors of the focal fish and setup the camcorder in this position.
3. Record all behavioral assays with the camera maintained in the same position with a full view of the specified tank to minimize unintended differences in behavioral scoring as a result of differential recordings.
NOTE: Avoid placing tanks in heavy trafficked areas and by doors as movement and sound will affect zebrafish behavior.
4. Establish unique JWatcher protocols²⁴ for scoring each behavioral assay. Refer to the JWatcher manual to learn how to create focal master files to assign behaviors to keystrokes and focal analysis files to summarize data collected.
NOTE: JWatcher is behavioral scoring software that allows for reliable and repeatable customized behavioral scoring²⁴.

5. Define a labeling scheme to track the date, fish number, and assay number for all behavioral assays (e.g., 'F5-B-123115' implying Fish 5, Boldness assay, on December 31st, 2015) and label the outside of the tank with tape in view and in focus of the camera. Keep and record this information in a lab notebook for quick reference and future quality control.
6. Set up an acclimation tank that will be used between all assays as well as directly after the individuals are removed from the housing rack. NOTE: The acclimation tanks and terminal tanks are small aquariums (40 cm x 20 cm x 25 cm) with gravel and a filter that closely resemble the housing rack.
7. After the assays are all completed, transfer the individual into a separate terminal tank that will serve as the final destination tank for all used focal individuals. Use a tank that is large enough to house a group of individuals for the remainder of their lives (e.g., 25 gallon). NOTE: It is very important to maintain consistent and similar water temperatures and conditions across all tanks. This includes housing, acclimation, and all testing tanks.

3. Conducting the Aggression Assay

1. Conduct the aggression assay in a 19 L rectangular tank measuring 30 cm x 15 cm x 10 cm. Outfit the tank in external opaque partitions surrounding three sides of the aggression tank, leaving the front exposed for viewing, to reduce undesirable outside stimuli. Divide the tank with external markings into four equal rectangular quadrants and fix a mirror permanently (with silicone caulk) within the lower left quadrant, inclined at 22.5° forming a right triangle against the far left side of the tank.
2. Ensure that the temperature of the water in the acclimation tank and aggression tank is within 2 °C of the housing rack. To do so, have fresh, aged water pre-warmed to 27.5-28.5 °C ready to add to the aggression tank. Please note that it is important to change the water in between each assay to eliminate unwarranted olfactory cues.
3. Transfer, by cupping, a focal fish from the housing rack to a separate acclimation tank where it will acclimate for 10 min. NOTE: It is important to reduce stimulus introduced to the fish through improper handling. Transfer individuals as previously described²⁷.
 1. Specifically, manually and carefully place a small, transparent plastic cup into the tank of interest. Without disrupting the fish as much as transferring by using a net, slowly scoop up the fish into the cup.
4. Ensure that the video camcorder is ready to record by confirming that the aggression assay tank (see step 3.1) is in focus and immediately before transferring the individual into the aggression tank, press record on the device. Transfer focal fish via cupping from acclimation tank to aggression tank. NOTE: It is important to record prior to the fish entering the aggression tank to track the exact time of entry.
5. Record the aggression tank for 10 min and 30 sec and then cup the focal fish from the aggression tank back into the acclimation tank.
6. When watching the recording, wait 30 sec after the fish has been introduced to the tank as an additional acclimation period before scoring behaviors.
7. Using the JWatcher scoring system defined for aggressive behaviors, quantify the following behaviors for 10 min: Time spent near mirror, number of mirror approaches, number of darts, number of attempted bites (opening mouth in direction of mirror), and number of lateral displays (dorsal, pectoral, anal, and caudal fin erection)⁹.
8. Score a single aggression assay at least twice, until the assay is scored with high agreement between iterations.

4. Conducting the Boldness Assay

1. Conduct the boldness assay in a small 1.5 L trapezoidal tank, measuring 15 cm tall x 26.5 cm top x 22.5 cm bottom x 6 cm width. Wrap opaque paper around all sides of the tank but leave the front exposed for viewing. Place a single, thin piece of tape external to the tank to demarcate the top from the bottom portion of the tank.
2. Ensure that the temperature of the water in the boldness tank is within 2 °C of the housing rack (see step 3.2)
3. Transfer the focal fish from the housing rack to the acclimation tank (see step 3.3) and let acclimate for 10 min.
4. Ensure that the camcorder is ready to record the boldness assay and, once ready to transfer the focal fish, press record on the device. Transfer the focal fish from the acclimation tank to the boldness tank and record for 8 min and 30 sec.
5. When watching the recording, allow for an additional 30 sec acclimation period after the fish is introduced to the tank before scoring.
6. Using JWatcher software, quantify boldness behaviors for 8 min. Specifically, quantify the following behaviors: Time near surface, latency to enter upper portion, number of transitions into the upper portion, and number of erratic movements (darts)²⁹.
7. Score a single boldness assay at least twice, until the assay is scored with high agreement between iterations.

5. Conducting the Shoaling Assay

1. Conduct the shoaling assay in a 76 L tank that has three compartments divided by two glass dividers sealed with silicone caulk (the resulting three compartments consist of two stimulus zones and one focal compartment). Externally, mark two preference zones designated 6.35 cm from each of the stimulus zones' glass divider, indicative of two body lengths³⁰. Apply opaque partitions externally around three sides of the shoaling tank, but leave the front exposed for viewing. NOTE: This preference zone can be customized to test for specific hypotheses for different sized species.
2. Depending on the nature of the study, select stimulus shoals to acclimate for at least 12 hr before testing and remain in the shoaling tank throughout the course of the experiment. NOTE: Stimulus shoals are collections of individual fish that are used to assess how the focal fish behaves in the presence or absence of these shoals. Different stimulus shoal combinations can be selected to test a number of diverse shoaling hypotheses. Be sure that the stimulus shoals are rotated between each stimulus zone to avoid side bias and are also allowed to acclimate for at least 12 hr before testing.
3. Transfer the focal fish into the acclimation tank as previously described (see step 3.3)
4. Transfer, by cupping, the focal fish from the intermediate acclimation tank to the central compartment in the shoaling tank.
5. Allow for a 10 min acclimation period before a 10 min live-scoring observation period. Alternatively, record the shoaling assays and score later using JWatcher. Fish are determined to be shoaling when they are within the preference zone in the chamber housing the target shoal.
6. Quantify time spent in each preference zone and time spent in the center volume.

7. As an additional descriptive measure, calculate 'strength of shoaling' (SoS). Assign a value between -1 and +1 where the magnitude describes how often an individual fish shoals.
8. Calculate the ratio of time spent shoaling divided by the area of the shoaling zones and subtract the ratio of time spent not shoaling and the area of the non-shoaling zones. Place this quantity over the sum of time spent in each zone divided by the volume of each zone individually. More information about this calculation can be found in a previous study¹⁴.

6. Data Quality Control

1. Analyze data using traditional data analysis tools³¹⁻³².
 2. Test for homogeneity of variance using nonparametric Levene's test for all behaviors. If a behavior is non-homogeneous perform a log-transformation³³.
 3. Confirm that there are no sequence effects (*i.e.*, that the randomization order did not affect the individuals' behavior) using a Kruskal-Wallis ANOVA on the homogeneous data³⁴.
 4. Confirm that there are no side biases for the shoaling assay by creating univariate general linear models on SoS indices with side as a fixed factor³⁵.
- NOTE: If there are little to no issues with the data, downstream analyses can be conducted. If there are issues, reconcile with additional transformations, rationalize with biologically plausible explanations, and/or increase sample sizes.

7. Analyze Data

1. Calculate two-tailed Spearman rank correlations³⁶ for all behaviors separately within the boldness and aggression assays.
2. Calculate intraclass correlation coefficients (ICC) as a two-way mixed model with consistency for behavioral assays that were conducted more than one time for a given individual³⁶. In this setting, the ICC is a measurement of how consistently a fish behaved in a given assay measured more than one time.
3. Preprocess boldness and aggression behaviors by centering and norming all behaviors to prepare for input to a principal components analysis (PCA)²⁵.
NOTE: Preprocessing the measurements by centering and norming is a vital step. Without this preprocessing step behaviors that occur with more frequency and more variably will excessively influence the interpretations of the extracted components. The process of centering and norming will remove this bias.
4. Perform a PCA with a correlation matrix to determine if boldness and aggression are linked together as a behavioral syndrome. Interpret behavioral loadings of each component significant at >0.6. See Budaev 2010²⁴ for a comprehensive guide for reporting PCA for behavioral studies. A scree plot will be informative on which eigenvectors should be kept for interpretation.
5. Using statistical software package such as R or SPSS^{31,32}, extract scores for each component for each individual. The scores represent how much the given individual's behavior can be explained by the particular component.
6. Calculate Spearman rank correlations between factor regression scores and the strength of shoaling³⁶.
NOTE: A behavioral syndrome is considered present if boldness and aggression behaviors load on the same component or, if any component with significant interpretable loadings correlates with strength of shoaling. Also, note that strength of shoaling is not included in the PCA to allow for better interpretations of the more complex behavioral quantifications (boldness and aggression). The PCA will orthogonally collapse boldness and aggression features into eigenvectors to maximize the amount of variation explained and is well suited for these complex measurements. Interpreting the resulting eigenvectors and regressing our strength of shoaling measurements on each of them gives a much more intuitive interpretation of how each of the complex behaviors (boldness and aggression) relate to a more well-defined binary shoaling measurement (*i.e.*, the fish is shoaling or not shoaling).

Representative Results

Depending on the nature of the study, and specific protocol employed, several distinct results are possible in a behavioral syndromes experiment. The following tables and figures, where indicated, are adapted from our previous study published in the journal *Behavioural Processes*¹⁴ and the journal *Zebrafish*¹⁷. When the proposal (as described above) is carried out in its entirety, two sets of results, 'within assay correlations' and 'between assay correlations,' are expected (**Figure 1**).

The first set of results describes within assay consistency. Specifically, the results describe how behaviors are correlated with each other within the boldness assay and the within the aggression assay separately. **Tables 1** and **2** describe what these correlations should look like for the boldness and aggression assays and the data presented are adapted from a previous study¹⁴. For the boldness assay, it is expected that number of transitions and time spent in the upper portion of the tank are positively correlated, but latency to enter the upper portion is negatively correlated. Representative results for individuals measured on the boldness axis of behavior in the boldness tank (see step 4.1) are given in **Table 1**. For the aggression assay, it is expected that bites, lateral displays, and time spent near the mirror are all correlated. Representative results for individuals measured on the aggression axis of behavior in the aggression tank (see step 3.1) are given in **Table 2**. Darts are not usually correlated with aggressive behavior although, in some cases, nonaggressive fish tend to dart (hence a negative correlation). Lastly, a Strength of Shoaling (SoS) measurement for the shoaling assay provides an individual-level measure of shoaling tendency. Shoaling is a highly repeatable behavior across a variety of shoaling assays when measured using SoS (ICC = 0.641)¹⁴ and several of our previous studies have confirmed shoaling behavior in zebrafish^{23,38}. Since all individuals will be measured for each of the behaviors, we can calculate Spearman correlations across these behaviors and expect some behaviors to be correlated, as identified for zebrafish in the representative results.

The second portion of the results investigates the presence of a behavioral syndrome. The results of the integrative analysis of all behaviors quantified in the boldness and aggression behavioral assays are summarized using a Principal Components Analysis (PCA). There should be two to four interpretable components based on high loadings for each behavior. If a single eigenvector that explains a good portion of the variation includes behaviors from both the boldness and aggression assays, then a behavioral syndrome has been observed. However, if behaviors from the boldness and aggression assays do not overlap on a single eigenvector, then the study does not describe a behavioral syndrome. Representative results are presented for the absence of a boldness-aggression behavioral syndrome (**Figure 2**). In this example, component 1 is most strongly representative of aggression behaviors while component 2 is most strongly associated with boldness behaviors. Because aggression and boldness behaviors are not represented by the same component, it can be concluded that there is the absence of a boldness-aggression behavioral syndrome (boldness vectors are roughly orthogonal to aggression vectors). Also, these behaviors are not influenced by sex because the distribution pattern is the same between males and females. To observe if boldness or aggression behaviors are associated with shoaling, the extracted regression scores for each fish are correlated with the fish's SoS measurement. It is important to ensure that any correlating components are actually describing an interpretable set of behaviors. If there are high absolute value Spearman correlations between any component and the shoaling measurement, then a shoaling behavioral syndrome is present. Regardless of the specific results, it is important to provide feasible biological interpretations to all observations. Representative results are provided for a description of a boldness-shoaling behavioral syndrome (**Table 3**)¹⁴. While the data presented represent typical results for zebrafish, they should not be interpreted as measurements of data quality. There are several reasons why the data will look different across different experiments such as species or population differences.

Figure 1. Proposed workflow for a boldness, aggression, and shoaling behavioral syndromes experiment. Typical measurements are listed for each assay. An outline of how to analyze the assays individually and then how to integrate the analyses is also listed. [Please click here to view a larger version of this figure.](#)

Figure 2. Representative results of a PCA indicating that a boldness-aggression behavioral syndrome is absent. The red vectors indicate behaviors which contribute to the component scores (left and bottom axes) and the points represent individual scores along each component (right and top axes). Males are represented by blue points and females are represented by green points. Note that 'erratic behavior' refer to 'darts' and 'aggression rate' can be computed by adding the number of bites and lateral displays divided by the total time interacting with the mirror. [Please click here to view a larger version of this figure.](#)

	Transitions	Latency	Darts			
	P	p	P	p	P	p
Surface Time	0.65	<0.001*	-0.68	<0.001*	0.42	0.007*
Transitions			-0.40	0.012*	0.33	0.041*
Latency					-0.47	0.002
* $\alpha < 0.05$						

Table 1: Boldness Spearman rank correlations in a novel tank assay. This table had been modified from Way *et al.*, 2015.

	Lateral Displays		Darts		Mirror Time	
	P	p	P	p	P	p
Bites	0.69	<0.001*	-0.10	0.547	0.63	<0.001*
Lateral displays			-0.06	0.735	0.66	<0.001*
Darts					-0.18	0.285
* $\alpha < 0.05$						

Table 2: Aggression Spearman rank correlations in a novel tank assay. This table had been modified from Way *et al.*, 2015.

	P	p
PCA Components		
Boldness	0.339	0.035*
Aggression	0.075	0.65
Dart	-0.012	0.944

Table 3: Using the PCA components to confirm the presence of a shoaling and boldness behavioral syndrome present in this population. This table had been modified from Way *et al.*, 2015.

Discussion

The protocol will determine if there are consistent associations in boldness, aggression, and shoaling behaviors in zebrafish. If there are consistent associations in a given population between any of these behaviors, then a behavioral syndrome is present. By studying a population's natural behavioral syndrome, researchers can have a more complete understanding of its behavioral dynamic, population structure, and possibly evolutionary history³. Furthermore, manipulating the environment that affects these behavioral syndromes, like in pharmacology⁶, toxicology⁷, behavioral genetics^{8,9}, and endocrinology¹⁰ studies, can shed light on how different factors might affect behavioral associations. The specific composition of the aggression-boldness-shoaling behavioral syndrome found in this protocol encompasses common behaviors well studied in the model organism zebrafish¹¹⁻¹³. While this methodology can be applied to any fish species, the wide application for using zebrafish may help with the interdisciplinary standardization of this protocol. Each assay described above can be more easily controlled and manipulated than other comparable techniques. For example, the aggression assay uses the inclined mirror to elicit a wider array of aggression responses rather than using a live or clay model, video stimulus, or vertical mirror image stimulus¹⁷. The behaviors measured in boldness assay are more easily quantified than in a t-maze or large open field test¹⁵. Lastly, the shoaling assay described can easily track an individual, which is essential for studying behavioral syndromes at the individual level, and can allow for unlimited combinations of target shoals to ask novel experimental questions. While this protocol can be altered to accommodate a variety of behavioral questions, the standardization of it may lead to further insight on how DNA, cellular mechanisms, cellular development, and introduced chemicals can affect individual and population behavioral syndromes.

In order to be confident that the observed associations are true, the protocol must be followed carefully. For the most part, the protocol is modifiable according to the specific hypothesis; however, there are several steps that must be performed to ensure confidence in the results. First, it is important that the individuals are housed in an appropriate tank, at optimal conditions (see protocol) and are fed properly. The water conditions should be consistent in housing racks, acclimation tanks, and test tanks. If the water is not consistent, the fish will take longer to acclimate to the conditions, and the behavioral measurements will not be properly captured. It is also important that water is recycled between boldness and aggression tanks so that artificial odors are not carried over between assays. Second, it is of utmost importance that all behaviors are scored properly. To ensure this, all assays are videotaped to allow for multiple viewings, and for several training opportunities. A single trained scorer can accurately measure fish behavior, but training does take some time. If a careful training period is ignored, then the confidence of the results of the behavioral study is low. A careful study with low sample sizes performed by a well-trained observer is more powerful than a study with high sample size performed by a poorly trained observer. Lastly, quality control of the collected data is also critical. The steps that are taken to randomize individuals, and remove biases should result in useable, reliable data, and data transformations are recommended for preprocessing according to the specific statistical test (see protocol). If these steps are not carefully done, the output of the analyses may not be reliably interpretable.

As previously mentioned, the technique is heavily modifiable according to the specific test hypotheses. There exist other behavioral measurements that can be investigated to be related in a behavioral syndrome among a number of diverse fish species³⁹⁻⁴¹. While the tenets

of the protocol remain the same, the specific questions can be easily altered to allow for diverse hypothesis testing. For example, a researcher may use the aforementioned protocol to test for the presence of an aggression and exploratory activity behavioral syndrome in convict cichlids (*Amatitlania nigrofasciata*). The question is related, but the study organism is different from the one described by the protocol. However, the general steps remain largely the same. Individuals must be randomized and tracked through a series of assays, with acclimation periods in between assays, the water must be consistent and fresh, and the scientist should be properly trained. One major difference is the behaviors that are being measured are likely to change according to the specific assay, and the behavioral associations may change according to the specific species and original environment.

The limitations of the protocol are linked with some of the traditional uncertainties of behavioral studies. Specifically, if the assays are scored unreliably then it is likely the interpretations of the findings are erroneous, and it would be difficult to identify this error. To overcome this limitation, it is possible that two well-trained scorers observe behaviors. To assess their reliability, calculate intraclass correlation coefficients on measurements applied on the same fish, and, if necessary, adjust for differences. Alternatively, if available, automatic tracking software such as Ethovision can be implemented and validated by a well-trained observer to increase throughput and accuracy⁴². Furthermore, there are different possible interpretations regarding the naming and scoring of the "boldness" behaviors. Other studies have termed the behavior described⁴³ and others have described the behavior as "exploratory"^{44,45}. In our work the behavior was described as "boldness" as it was measuring behavior in an environment unfamiliar to the focal individual. However, while the term may be subject to alternative interpretations, this does not affect the protocol or the analysis. Additionally, while we expect measurements within boldness and aggression assays to be strongly correlated within a population, there is likely to be some instances of low to no correlation of some behaviors. This limitation is overcome by the strength of the PCA, because it keys in on important sources of variability and, even if measurements are not correlated, the analysis will extract behavioral variation consistent in the collected data. Lastly, as is the case with all scientific methods, if the protocol is universally adopted and is consistently carried out by several labs, and there is some unforeseen, unmeasured confounding introduced by the protocol, then this potential deleterious element persists in the literature, and it becomes difficult to dismantle. Pharmacologically confirming that a representative set of assays elicit the intended behavioral response requires a more thorough understanding of behavioral, neurological and hormonal responses. Behavioral syndromes can help explain the basis of behavior, but this laboratory limitation may be able to be more regularly addressed in future studies. Nevertheless, results have been provided that validates the use of this protocol in zebrafish, and, with proper modifications, the protocol can be extended to a variety of hypotheses in a number of different fish species. By carefully following a detailed protocol for the housing, selection and testing of various behaviorally parameters will enable researchers to make more specific comparisons across a wide array of studies.

Disclosures

The authors (GW and SPM) have no competing financial interests or conflicts of interest.

Acknowledgements

This work was supported by a Howard Hughes Medical Institute Education Grant and an internal grant from the Saint Joseph's University chapter of Sigma Xi. We would also like to thank the three anonymous reviewers who helped strengthen the protocol and interpretations.

References

- Huntingford, F. The relationship between anti-predator behaviour and aggression among conspecifics in the three-spined stickleback, *Gasterosteus aculeatus*. *Anim Behav.* **24**, 245-260 (1976).
- Réale, D., Reader, S. M., Sol, D., McDougall, P. T., & Dingemanse, N. J. Integrating animal temperament within ecology and evolution. *Biol Rev.* **82** (2), 291-318 (2007).
- Sih, A., Bell, A., & Johnson, J. C. Behavioral syndromes: an ecological and evolutionary overview. *Trends Ecol Evol.* **19** (7), 372-378 (2004).
- Conrad, J. L., Weinersmith, K. L., Brodin, T., Saltz, J. B., & Sih, A. Behavioural syndromes in fishes: a review with implications for ecology and fisheries management. *J Fish Biol.* **78** (2), 395-435 (2011).
- Wolf, M., & Weissing, F. J. Animal personalities: consequences for ecology and evolution. *Trends Ecol Evol.* **27** (8), 452-461 (2012).
- Langheinrich, U. Zebrafish: a new model on the pharmaceutical catwalk. *BioEssays.* **25** (9), 904-912 (2003).
- Dai, Y.-J., Jia, Y.-F., *et al.* Zebrafish as a model system to study toxicology: Zebrafish toxicology monitoring. *Environ Toxicol Chem* **33** (1), 11-17 (2014).
- Norton, W., & Bally-Cuif, L. Adult zebrafish as a model organism for behavioural genetics. *Neuroscience.* **11** (1), 90 (2010).
- Gerlai, R. Zebra fish: an uncharted behavior genetic model. *Behav Genet.* **33** (5), 461-468 (2003).
- Dziewieczynski, T. L., Campbell, B. A., Marks, J. M., & Logan, B. Acute exposure to 17 α -ethinylestradiol alters boldness behavioral syndrome in female Siamese fighting fish. *Horm Behav.* **66** (4), 577-584 (2014).
- Miklósi, Á., & Andrew, R. J. The Zebrafish as a Model for Behavioral Studies. *Zebrafish.* **3** (2), 227-234 (2006).
- Zebrafish protocols for neurobehavioral research*. Humana Press: New York, (2012).
- Moretz, J. A., Martins, E. P., & Robison, B. D. Behavioral syndromes and the evolution of correlated behavior in zebrafish. *Behav Ecol.* **18** (3), 556-562 (2007).
- Way, G. P., Kiesel, A. L., Ruhl, N., Snekser, J. L., & McRobert, S. P. Sex differences in a shoaling-boldness behavioral syndrome, but no link with aggression. *Behav Process.* **113**, 7-12 (2015).
- Toms, C. N., Echevarria, D. J., & Jouandot, D. J. A methodological review of personality-related studies in fish: Focus on the shy-bold axis of behavior. *J Comp Psychol.* **23**, 1-25 (2010).
- Rowland, W. J. Studying visual cues in fish behavior: A review of ethological techniques. *Environ Biol Fish.* **56** (3), 285-305 (1999).
- Way, G. P., Ruhl, N., Snekser, J. L., Kiesel, A. L., & McRobert, S. P. A Comparison of Methodologies to Test Aggression in Zebrafish. *Zebrafish.* **12** (2), 144-151 (2015).
- Moss, S., Tittaferante, S., *et al.* Interactions between aggression, boldness and shoaling within a brood of convict cichlids (*Amatitlania nigrofasciatus*). *Behav Process* **121**, 63-69 (2015).

19. Ruhl, N., Kiesel, A. L., McRobert, S. P., & Snekser, J. L. Behavioural syndromes and shoaling: connections between aggression, boldness and social behaviour in three different Danios. *Behaviour*. **149** (10-12), 1155-1175 (2012).
20. Larson, E. T., O'Malley, D. M., & Melloni, R. H., Jr. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behav Brain Res*. **167** (1), 94-102 (2006).
21. McRobert, S., & Bradner The influence of body coloration on shoaling preferences in fish. *Anim Behav*. **56** (3), 611-615 (1998).
22. Ruhl, N., & McRobert, S. The effect of sex and shoal size on shoaling behaviour in Danio rerio. *J Fish Biol*. **67** (5), 1318-1326 (2005).
23. Snekser, J., Ruhl, N., Bauer, K., & McRobert, S. The influence of sex and phenotype on shoaling decisions in zebrafish. *J Comp Psychol*. **23**, 70-81 (2010).
24. Blumstein, D. T., Evans, C. S., & Daniel, J. C. *JWatcher*. Available online at: <<http://www.jwatcher.ucla.edu/>> (2006).
25. Budaev, S. V. Using Principal Components and Factor Analysis in Animal Behaviour Research: Caveats and Guidelines. *Ethology*. **116** (5), 472-480 (2010).
26. Paciorek, T., & McRobert, S. Daily variation in the shoaling behavior of zebrafish Danio rerio. *Curr Zool*. **58** (1), 129-137 (2012).
27. Haahr, M. *Sequence randomiser*. Available online at: <www.random.org/lists/> (1998).
28. Wright, D., & Krause, J. Repeated measures of shoaling tendency in zebrafish (Danio rerio) and other small teleost fishes. *Nat Protoc*. **1** (4), 1828-1831 (2006).
29. Cachat, J., Stewart, A., *et al*. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nat Protoc*. **5** (11), 1786-1799 (2010).
30. Croft, D. P., Krause, J., Couzin, I. D., & Pitcher, T. L. When fish shoals meet: outcomes for evolution and fisheries. *Fish Fish*. **4**, 138-146 (2003).
31. IBM Corp. *IBM SPSS Statistics for Mac OS*. IBM Corp: Armonk, NY, at <<http://www.ibm.com/analytics/us/en/technology/spss/spss.html>> (2011).
32. *R: A language and environment for statistical computing*. at <<http://www.R-project.org>> R Foundation for Statistical Computing: Vienna, Austria, (2013).
33. Brown, M. B., & Forsythe, A. B. Robust Tests for the Equality of Variances. *J Am Stat Assoc*. **69** (346), 364 (1974).
34. Kruskal, W. H., & Wallis, W. A. Use of Ranks in One-Criterion Variance Analysis. *J Am Stat Assoc*. **47** (260), 583-621 (1952).
35. Nelder, J. A., & Wedderburn, R. W. M. Generalized Linear Models. *J Roy Stat Soc A Sta*. **135**, 370-384 (1972).
36. Iman, R. L., & Conover, W. J. A distribution-free approach to inducing rank correlation among input variables. *Commun Stat Simulat*. **11** (3), 311-334 (1982).
37. Fleiss, J. L., & Cohen, J. The Equivalence of Weighted Kappa and the Intraclass Correlation Coefficient as Measures of Reliability. *Educ Psychol Meas*. **33** (3), 613-619 (1973).
38. Ruhl, N., McRobert, S. P., & Currie, W. J. S. Shoaling preferences and the effects of sex ratio on spawning and aggression in small laboratory populations of zebrafish (Danio rerio). *Lab Animal*. **38** (8), 264-269 (2009).
39. Bell, A. M. Behavioural differences between individuals and two populations of stickleback (*Gasterosteus aculeatus*). *J Evolution Biol*. **18** (2), 464-473 (2005).
40. Wilson, A. D. M., & Godin, J.-G. J. Boldness and behavioral syndromes in the bluegill sunfish, *Lepomis macrochirus*. *Behav Ecol*. **20** (2), 231-237 (2009).
41. Brodin, T. Behavioral syndrome over the boundaries of life--carryovers from larvae to adult damselfly. *Behav Ecol*. **20** (1), 30-37 (2008).
42. Noldus, L. P., Spink, A. J., & Tegelenbosch, R. A. EthoVision: a versatile video tracking system for automation of behavioral experiments. *Behav Res Methods Instrum Comput*. **33** (3), 398-414 (2001).
43. Cote, J., Fogarty, S., Weinersmith, K., Brodin, T., & Sih, A. Personality traits and dispersal tendency in the invasive mosquitofish (*Gambusia affinis*). *Proc R Soc B*. **277** (1687), 1571-1579 (2010).
44. Fraser, D. F., Gilliam, J. F., Daley, M. J., Le, A. N., & Skalski, G. T. Explaining leptokurtic movement distributions: intrapopulation variation in boldness and exploration. *Am Nat*. **158** (2), 124-135 (2001).
45. Dingemanse, N. J., Wright, J., Kazem, A. J. N., Thomas, D. K., Hickling, R., & Dawnay, N. Behavioural syndromes differ predictably between 12 populations of three-spined stickleback. *J Anim Ecol*. **76** (6), 1128-1138 (2007).