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A Comparison of Methodologies to Test Aggression in Zebrafish

Gregory P. Way,[1] Nathan Ruhl,[2] Jennifer L. Snekser,[3] Alexis L. Kiesel,[1] and Scott P. McRobert[1]

Abstract

Aggression is a psychological construct that is commonly used to classify zebrafish behavior. Aggression is a complex trait that can be difficult to accurately measure. The literature on fish behavior describes many different methodologies to examine aggression, which, we believe, have not been compared in a formal manner. In this study we observed 19 individual zebrafish (*Danio rerio*) and quantified bites, lateral displays, charges, darts, and time near the stimulus in six common assays used to measure aggression. The methodologies included an inclined mirror assay, two flat mirror assays with different acclimation periods, a live conspecific assay, a clay model stimulus assay, and a video recording assay. Our results indicate high repeatability in most aggressive behaviors over time, which confirms the value of each assay to measure personality. However, our results also indicate significant differences between the assays. Specifically, assays using a flat mirror or live conspecific as a stimulus for aggression elicited more attempted bites than an inclined mirror, a clay model stimulus, or a video recording stimulus. Furthermore, the inclined mirror stimulus provoked more darts than any other assay. The results suggest the need for researchers to consider specific research goals when selecting the appropriate stimulus to provoke aggression in zebrafish.

Introduction

When an individual animal expresses similar behavior patterns across different contexts, it is said to have a personality.[1] In the past decade, research on animal personality has become more common, and with this increase there comes a need for clear and consistent terms and definitions.[1,2] To discern components of animal personality, researchers have attempted to isolate and define consistent behavioral axes.[3] These axes are commonly used to group animals according to their behavioral displays, and are ultimately useful for identifying personality types within a species or group. These behavioral measures have been investigated in a variety of fauna[4] and one of the most commonly measured axes is aggression.[3]

Various definitions of aggression have emerged throughout the literature, which complicates the qualification of aggressive displays. Lorenz (1974) defines aggression as fighting behavior directed against a separate individual of the same species. Others specify a distinction between Lorenz's aggression, sometimes referred to as agonistic behavior, and aggression against an individual of another species.[5] However, the expression of aggression can be considered completely distinct from a predator–prey relationship and further distinctions can be made according to the ecological situation.[5,6] For aggression to be properly defined, it is necessary to assume the mental state, or intent, of an individual. However, this is precisely what an objective viewer attempts to avoid when scoring behavior. Furthermore, aggression is a very complex behavior, which involves a cascade of genetic, neurophysiological, hormonal, and behavioral inputs that continually develop over time, are impacted by experiences, and vary across species.[7–10] Because of observational difficulties and complexities of expression, aggression can be a difficult behavior to define, isolate, and then measure in the laboratory.

There is a growing body of multi-disciplinary literature on zebrafish aggression. For instance, aggression has already been investigated in zebrafish from a variety of perspectives including neurogenomics,[11] neurophysiology,[12] psychiatric diseases,[13] toxicology,[14] pharmacology,[15] and animal behavior.[16–18] Because of this burgeoning research question, it is critical to investigate how the behavioral manipulations are performed and to compare the techniques used. Similar comparisons of other behavioral methodologies have been performed[19,20] but not a systematic and exclusive study on assays to measure aggression has been conducted. In general,

[1]Department of Biology, Saint Joseph's University, Philadelphia, Pennsylvania.
[2]Department of Biological Sciences, Rowan University, Glassboro, New Jersey.
[3]Department of Biology, LIU Post, Brookville, New York.

zebrafish aggression is measured by exposing the animal to specific stimuli and then quantifying the response. Several different types of assays have been developed that rely on diverse forms of stimuli; examples include mirror-image stimuli (MIS), live conspecific stimuli, clay model stimuli, and video stimuli.[21] In general, the same set of responses are measured in each kind of assay, and include bites, lateral displays, charges, and darts.[8] Early studies of fish aggression used a clay model stimuli[22] and the behavior of live conspecifics was qualified based on ''aggressive'' displays relative to the stimuli.[23,24] Recent technological advances have resulted in video stimuli becoming more popular than the use of models because phenotypic and behavioral parameters of the stimuli can be easily and quickly manipulated.[25,26] Despite the technological advances in video playback, many studies still use a strategically placed mirror to elicit an aggressive response.[27,28]

The aforementioned studies of zebrafish aggression nestle within a larger investigation of animal personality. Zebrafish are social animals, and in their interactions with conspecifics, they are capable of displaying a range of measurable behaviors. These factors make them excellent subjects for exploring animal personality, and a growing body of research exists around this topic. Specifically, recent work has explored shoaling tendencies, exploratory behavior, dominance, the bold/shy continuum, and aggression.[29] An aggressive individual, in animal personality theory, will behave consistently in different environmental situations, which may have profound effects on population dynamics and the evolution of species.[1,16] Furthermore, behavioral genetics has also been investigated using zebrafish as a model to exploregenetic correlation in behaviors,[30,31] which may be inherited maternally.[32] Therefore, the purpose of our present study is to confirm the persistence of aggression in different contexts, and to compare the differing methodologies used to investigate zebrafish aggression. The literature is strong in support of both instances, which allows for the concurrent investigation.

In the present study, we tracked 19 individual zebrafish through a battery of six distinct aggression assays as well as a novel tank assay lacking an aggressive stimulus, to serve as a control. The assays included three different mirror-image stimulus (MIS) assays (response to a novel tank with a flat mirror, response to an acclimated tank with a novel flat mirror, and response to a mirror inclined at 22.5°), a live conspecific assay, a clay model assay, and a video recording assay. The same behaviors were quantified across all assays and were analyzed to determine (i) if there was consistency within individual behaviors across assays and, (ii) if there were any consistent differences in behavioral responses by assay. Our data should assist researchers in making informed decisions regarding the appropriate assay for their aggression studies.

Materials and Methods

Animal care and housing

Wildtype zebrafish (*Danio rerio*) were obtained as adults from a local distributor (Seven Star Tropical Fish, Philadelphia, PA). A pet store population was used with intent to increase phenotypical variation, which would increase individual behavioral differences. All fish were allowed to acclimate for about 1 month and were initially maintained in a 76 L aquarium at 27.5°C–28.5°C, under a 12:12 light/dark cycle, and fed flake food daily. The methodologies involved in the acquisition, care, and study were approved by the Saint Joseph's University IACUC.

Before aggression assays, individual fish were randomly selected from the lab population and transferred to labeled individual chambers for 1 day. The individual chambers were constructed similar to those used by Wright and Krause[33] to facilitate accurate identification of individuals over time. Specifically, the individual chambers, which housed each individual fish, were all placed within an even larger water bath; as to maintain a consistent environment for all focal fish. Each translucent plastic individual chamber had small holes drilled in the sides to allow water to flow with the water bath. The individual chambers also contained black gravel to facilitate cycling of organic waste and a small plant to increase habitat complexity. The water bath was heated to 27.5°C–28.5°C, and water and organic waste were cycled using standard aquarium filters. Additionally, nonfocal fish were allowed to swim freely in the open space between the individual chambers, which may have provided olfactory and visual stimulation to the fish in the individual chambers with a goal of reducing isolation effects. Fish were kept on a 12:12 light/dark cycle, and all assays were performed between 09:00 and 13:00 to avoid circadian effects.[34] Focal fish were tracked through all assays within 3 weeks of being confined to an individual chamber.

Assays

The six aggression assays examined here (three versions of the mirror-image stimulus assay, the live conspecific assay, the clay model assay, and the video recording assay) represent common methods utilized by researchers to quantify aggression. While the differences between the assays are subtle, particularly subtle between the mirrored assays, they may cause fish to behave differently and thus, the interpretations of aggressive behavior will be different. To assess these differences, focal fish were tracked through a succession of six distinct aggression assays and a novel tank control (seven assays total). Individuals were tested in no more than one assay per day and the order in which each fish went through the assays was determined using a random number generator. To reduce transfer effects, fish were moved between individual chambers and acclimation tanks by cupping (transferring them in a small container), and were allotted 10 min to acclimate before each assay.[33] Fish were cupped again from the acclimation tank to the indicated assay.

Behaviors. During each assay, the following behaviors were recorded: number of bites, lateral displays, darts, charges, and the time spent directly interacting with the stimulus. The quantified behaviors are well established and commonly studied in the zebrafish literature.[8] Bites are described when the focal fish approaches the stimulus and rapidly extends an open mouth in the specified direction. A lateral display is observed when the focal fish approaches the stimulus with dorsal, pectoral, anal, and caudal fin erected and displays while turning to one side. A charge is defined by a rapid, and sometimes spontaneous, acceleration toward the stimulus. Similarly, darts are defined by a rapid acceleration anywhere except toward the stimulus. Time spent interacting

with the stimulus was only measured when the focal fish was in direct contact with the partition that separated the fish from the stimulus (denoted the contact zone). Finally, upon completion of each assay, the focal fish was returned to the same individual chamber for continued tracking.

Assay 1: control. Focal fish were exposed to a novel (bare bottom) 18.9 L rectangular tank lacking an aggression stimulus, and their behavior was recorded for a period of 480 s after a 30 s acclimation period. This assay was scored before the mirror introduction in assay 3.

Assay 2: inclined mirror-image stimulus (MIS). Assay 2 was conducted in a 18.9 L rectangular tank with a mirror (10.5 × 6.5 cm) fixed at a position inside the lower left corner of the tank at a 22.5° angle.[20] Only the front of the tank was transparent, which allowed for recording of the interaction of the focal fish with the mirror stimulus. Behaviors were quantified for 480 s.

Assay 3 and 4: alternative mirror-image stimuli (MIS). In assay 3, focal individuals were introduced to an 18.9 L bare tank and allowed to acclimate for 30 min. Once acclimated, an external flat mirror (20 × 22 cm) was exposed and behaviors were quantified for 480 s. In assay 4, focal individuals were introduced to an 18.9 L tank, with a mirror (20 × 22 cm) already exposed, and allowed to acclimate for 30 s. Once acclimated, behaviors were quantified for 480 s. The side the mirror was positioned on was randomized between assays to avoid any bias. Both assays were modified from previous studies.[27]

Assay 5: live conspecific stimulus. Size-matched individuals were allowed to acclimate in a glass-partitioned 18.9 L tank for 30 min before each assay. The tank was partitioned so that 1/3 housed the stimulus fish and 2/3 was allotted for measuring aggression in the focal fish. Tank orientation was frequently switched to account for unintentional biases. Behaviors were quantified for 480 s following a 30 s acclimation period. This assay is a common procedure adapted from several studies.[24]

Assay 6: clay-model stimulus. Modified from several studies investigating aggression in fish, this stimulus included a constructed size- and color-matched clay model.[22] Several models were constructed, but only one model was used for all replicates. As in assay 5, the clay model tank was partitioned with 1/3 of the tank for the stimulus and 2/3 of the tank for the focal fish. In this case, the clay model was suspended into an empty (dry) stimulus area by a thin string with a piece of paper attached to it well above the level of the tank. A strategically positioned fan gently blew a stream of air against the paper, causing simulated movement of the clay model in the stimulus partition. This motion simulated aggressive actions toward the focal fish such as head-on approaches and lateral turns. Tank orientation was frequently switched to account for unintentionally introduced variables. Behaviors were quantified for 480 s following a 30 s acclimation period.

Assay 7: video stimulus. A random nonfocal fish was video recorded performing aggressive acts in front of a mirror.[25,26] The film was edited into a 9 min long film of constant aggressive acts presented to the focal fish via a Macintosh computer screen. This assay was conducted exactly the same as assay 4, but instead of introducing a mirror stimulus against the glass, the computer screen was pressed to the glass. Behaviors were quantified for 480 s following a 30 s acclimation period.

Software and statistics

All assays were recorded using a Hitachi camera with a Nikon lens. The recordings were scored two to four times by a single reviewer (GPW) to ensure accuracy. The behaviors were quantified with JWatcher version 1.0 for Mac[35] and data was analyzed with SPSS,[36] JMP,[37] and SigmaPlot.[38] Sequence effects were investigated through a nonparametric Levene's test to confirm homogeneity, and then by a Kruskal–Wallis ANOVA. To investigate the presence of a consistent aggression personality in the different contexts presented by each of the assays, intraclass correlation coefficients (ICCs) were obtained from each of the five quantified behaviors. ICCs were also calculated for correlations between all five quantified behaviors. The ICCs were run with consistency on a mixed model to reduce any potential biases introduced by the differential nature of each assay and were calculated and reported according to Lessells and Boag.[39]

Mean differences in bites, lateral displays, darts, and charges were compared through Steel-Dwass nonparametric multiple comparisons. The ability of each assay to elicit a range of behavioral responses was investigated by Tukey's Honestly Significant Differences multiple comparisons statistic on variance measurements. To obtain the data in the proper form for the variance differences, Bartlett and Kendal transformations were performed according to a procedure outlined in Levy.[40] Within assay relationships were calculated with Spearman correlations.

Results

Repeatability of aggression

Randomization efforts were successful; no sequence effects were detected between any of the assays performed at any time period. The frequency of lateral displays was fairly repeatable between assays ($ICC = 0.358$, $F = 1.558$) while the frequency of charges was only slightly repeatable ($ICC = 0.096$, $F = 1.106$; Table 1). Bites ($ICC = 0.561$, $F = 2.276$) and time near object ($ICC = 0.518$, $F = 2.074$) were moderately repeatable (Table 1). Darts were the most repeatable behaviors between the six assays ($ICC = 0.721$, $F = 3.584$; Table 1).

Table 1. Repeatability of Aggressive Behaviors

Behavior	ICC	F *test*	p-*Value*
Bites	**0.561**	**2.276**	**0.00587**
Lateral displays	0.358	1.558	0.08881
Charges	0.096	1.106	0.35989
Darts	**0.721**	**3.584**	**0.00003**
Time near object	**0.518**	**2.074**	**0.01305**

Repeatability of behavior calculated across all assays excluding the control. Repeatability is reported as ICCs on a mixed model with consistency. *p*-Value assesses *F* test difference from zero.
Bold text indicates a significant intra-class correlation.
ICC, intraclass correlation coefficient.

Table 2. Repeatability of Aggression Correlations

	Displays	*Charges*	*Darts*	*Time*
Bites				
ICC	**0.4577**	0.0593	−0.148	**0.7977**
F	**1.8441**	1.0630	0.8710	**4.9944**
p	**0.0006**	0.3730	0.7679	**0.0000**
Displays				
ICC		**0.3283**	−0.002	**0.2977**
F		**1.4890**	0.9983	**1.4240**
p		**0.0177**	0.5033	**0.0308**
Charges				
ICC			**0.2773**	0.0333
F			**1.3838**	1.0344
p			**0.0428**	0.4290
Darts				
ICC				−0.085
F				0.9214
p				0.6679

Repeatability of correlations calculated across all assays excluding the control. Repeatability is reported as ICCs on a mixed model with consistency. *p*-Value assess *F* test difference from zero.
Bold text indicates a significant intra-class correlation.

Correlations across all assays were variably reliable. Correlations between bites and lateral displays ($ICC = 0.458$, $F = 1.814$), and bites and time spent near the stimulus ($ICC = 0.797$, $F = 4.994$) were reliable (Table 2). Lateral display correlations with charges ($ICC = 0.324$, $F = 1.489$) and time near stimulus ($ICC = 0.298$, $F = 1.424$) were reliable as well (Table 2). Charges and darts correlation were also reliable ($ICC = 0.277$, $F = 1.383$; Table 2). Time spent near the mirror was very nearly autocorrelative with bites in every assay ($ICC = 0.798$, $F = 4.994$). Therefore, time near mirror was omitted from the assay differences investigation.

Assay differences

Mean response differences. There were considerable differences between assays in eliciting a mean behavioral response. Specifically, bites were divided into two subsets of high and low expression, with assay 2, 6, and 7 loading with the control in the low expression group, and with assay 3, 4, and 5 loading in the high expression group (Fig. 1). Number of lateral displays grouped less clearly, but there was low expression in assay 6, 7, and the control, moderate expression in assay 2 and 3, and the highest expression in assay 4 and 5 (Fig. 1). Darts loaded into two distinct subsets as well. Assays 3, 4, and 5 elicited a lower amount of darts than assay 2 (Fig. 1). Lastly, there were no differences detected in charge behaviors (Fig. 1). Bites were also the most common behavior for the individuals to express.

Range response differences. There were considerable differences between the assays in terms of eliciting a spectrum of behavioral responses. Bites loaded similar to how they did for mean response differences. Assay 2 was not

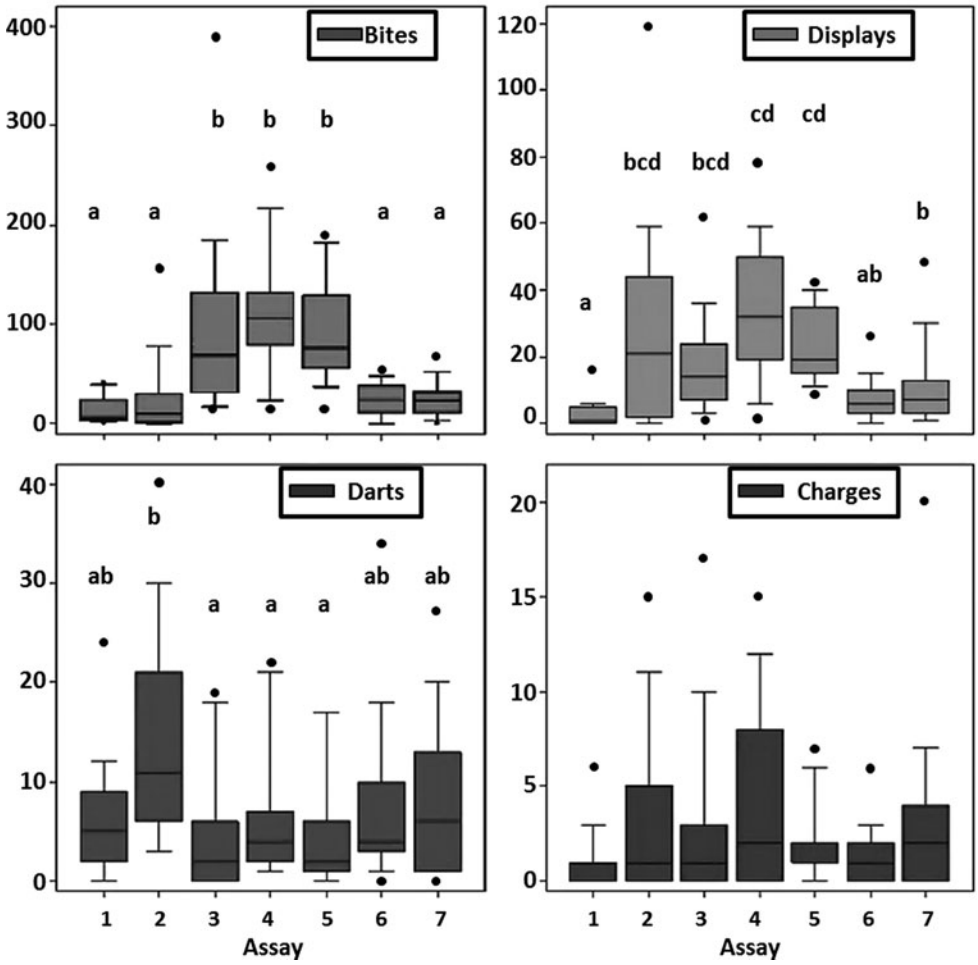

FIG. 1. All mean differences obtained by Steel-Dwass nonparametric multiple comparisons test. Alphabetic markers indicate homogenous subsets. There are no differences in charges behavior. The assays are represented in the following way: (1) control, (2) inclined mirror, (3) flat mirror with 30 min acclimation, (4) flat mirror with 30 s acclimation, (5) live conspecific stimulus, (6) clay model, and (7) video recording.

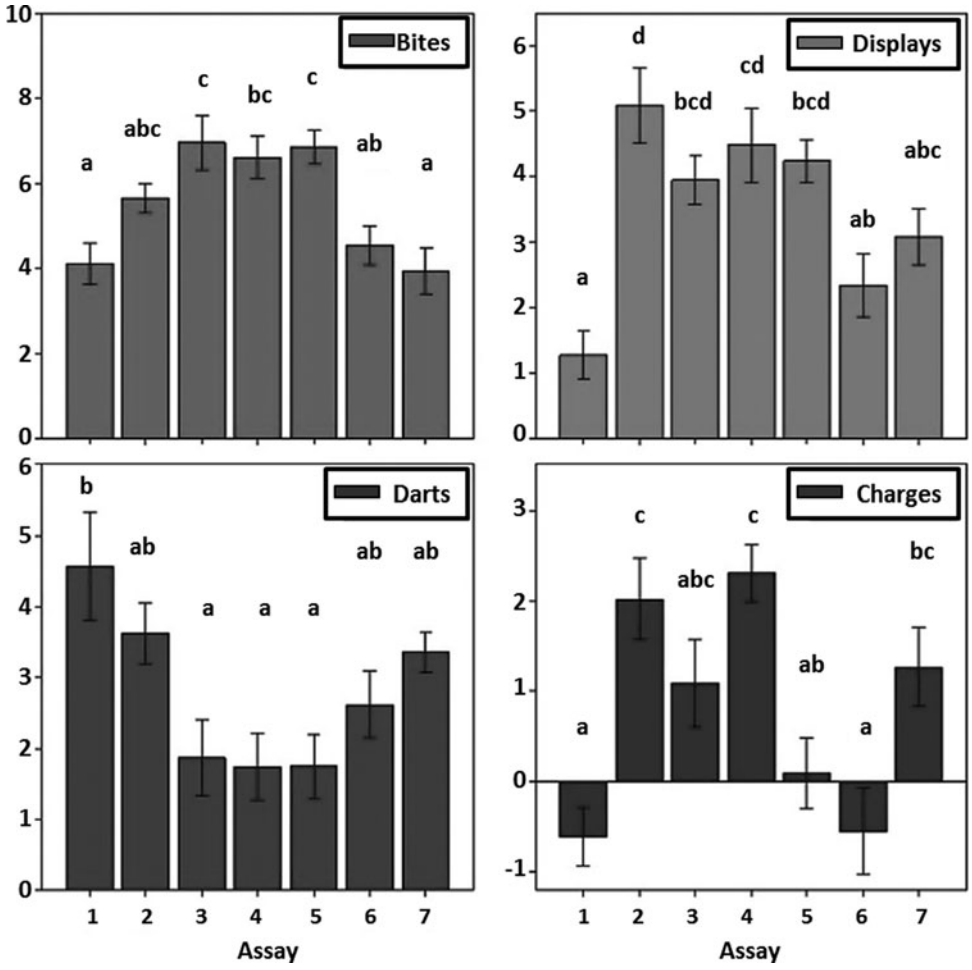

FIG. 2. All variance differences obtained by Bartlett and Kendal transformations and Tukey's Honestly Significant Differences multiple comparisons test (according to Levy 1975). Alphabetic markers indicate homogenous subsets. The scales are log transformed variance values and error bars represent ± standard error of the variance mean. (1) control, (2) inclined mirror, (3) flat mirror with 30 min acclimation, (4) flat mirror with 30 s acclimation, (5) live conspecific stimulus, (6) clay model, and (7) video recording.

exclusive to any subset, but assay 3 and 5 were different from assay 6, assay 7, and the control (Fig. 2). Assay 2 elicited the largest range of lateral displays while assays 6, 7, and the control elicited a lower range of lateral displays, and assays 3, 4, and 5 composed their own subset of intermediate variance (Fig. 2). We observed a low range of darts in assays 3, 4, and 5, and no differences for assay 2, 6, and 7. Interestingly, the control assay, an empty novel tank assay, elicited the highest range of dart expression (Fig. 2). The control and assay 6 elicited the lowest range of charges, whereas assay 2 and 4 had the highest range (Fig. 2).

Within-assay correlation differences. There were some differences observed when comparing the repeatable behavioral correlations across assays. Bites and lateral displays were correlated, and time near the stimulus and bites were correlated,

Table 3. Select Within-Assay Behavioral Correlations

		Bites vs. displays	*Bites vs. charges*	*Displays vs. charges*	*Charges vs. darts*	*Time vs. bites*	*Time vs. displays*
Assay 1	*r*	**0.57**	0.44	−0.09	0.18	**0.90**	0.43
Control	*p*	**0.0117**	0.0569	0.7223	0.4702	**0.0000**	0.0672
Assay 2	*r*	**0.88**	**0.60**	**0.72**	0.36	**0.87**	**0.88**
22.5°	*p*	**0.0000**	**0.0062**	**0.0050**	0.1280	**0.0000**	**0.0000**
Assay 3	*r*	**0.58**	0.21	0.44	0.27	**0.84**	**0.55**
30 min	*p*	**0.0086**	0.3815	0.0568	0.2620	**0.0000**	**0.0151**
Assay 4	*r*	**0.67**	0.25	0.37	**0.51**	**0.80**	**0.67**
30 s	*p*	**0.0018**	0.3109	0.1171	**0.0261**	**0.0000**	**0.0017**
Assay 5	*r*	**0.74**	0.34	0.13	0.04	**0.80**	**0.80**
Live	*p*	**0.0030**	0.1609	0.5826	0.8786	**0.0000**	**0.0000**
Assay 6	*r*	**0.52**	0.38	**0.57**	0.19	**0.78**	**0.70**
Clay	*p*	**0.0219**	0.1108	**0.0101**	0.4304	**0.0000**	**0.0009**
Assay 7	*r*	**0.76**	0.37	0.42	0.19	**0.81**	**0.73**
Video	*p*	**0.0001**	0.1173	0.0711	0.4436	**0.0000**	**0.0004**

Spearman correlations are calculated across all assays including the control. *Bold* denotes significant correlations with $\alpha = 0.05$.

across all assays, including the control (Table 3). Time spent near the stimulus and lateral displays were also correlated in each assay, except for the control (Table 3). Assay 3, 5, and 7 had similar correlation patterns (Table 3). Additionally, most of the selected aggression behaviors, with the exception of charges and darts, were correlated in assay 2 (Table 3).

Discussion

The goals of this study were to confirm the consistency of aggressive behaviors in different assays and to compare the assays commonly used to study zebrafish aggression. Supporting our first aim, individual zebrafish reliably demonstrated consistent aggressive behaviors, thus confirming the presence of persistent aggressive personalities. The results also indicated that between all aggression assays correlations were dependably maintained. Our reliability scores for individual behaviors and behavioral correlations were consistent with the accepted average estimate of 0.37.[41] Charges, however, were the least repeatable behavior and, despite being defined as an aggressive action,[8] they failed to predict an individual's level of aggression consistently. Thus, it may be possible that charges either do not measure aggression accurately, or they are a behavioral response independent of aggression motivations. The consistency of most aggression behaviors, and the large differences observed between the zebrafish, indicates that there is some force, whether it is environmental constraint, early experience, hormones, gene pleiotropy, or some combination, differentially impinging upon the expression of aggression within a population.[42–44]

In comparing commonly used aggression assays our results demonstrated three distinct subsets of assays separated by differential behavioral elicitation. The two flat mirror assays (assays 3 and 4) and the live conspecific assay (assay 5) had similar behavioral expressions and loaded to a distinct subset characterized by high bites, high displays, and low darts. A second subset consisted of the control (assay 1), the clay model stimulus (assay 6), and the video recording stimulus (assay 7) and was characterized by low bites, low displays, and an intermediate amount of darts. Lastly, the third subset, composed of the inclined MIS assay (assay 2), was characterized by low bites, high displays, and high darts.

In considerations of within-subset differences, the most similar assays in behavioral elicitation were the two flat mirror assays and the live conspecific assay, which all exhibited similar expression patterns of bites, lateral displays, and darts. The only differences arose when comparing the range of charges and range of displays elicited by each assay. The 30-s acclimation period (assay 4) resulted in a somewhat higher range of charges and displays than the 30-min acclimation period (assay 3), although they were not classified as distinct subsets on the behavioral measures. The live conspecific assay elicited even less of a charge distribution than the two flat mirror assays. Since the only construction differences between the two MIS assays was an increased acclimation time it is possible that the 30-min assay led to a territorial response, whereas the 30-s assay led to an aggressive response to the novelty of the tank and mirror.[27] However, since the only differences were marginal it can be inferred that either a 30-min acclimation period is not long enough to elicit a strong territorial response or a territorial response in zebrafish is not different than the general aggression response. It also may be possible that territorial behaviors are suppressed in this population since natural stressors are altered in a laboratory setting; which has been previously observed to influence behavior.[45]

The clay model assay (assay 6) and video stimulus assay (assay 7) were also highly similar, and were frequently grouped with the control assay (assay 1). Even though they did not express many more differences than an empty control tank, the clay model and video recording stimuli did clearly elicit some form of aggressive response. The main differences were in eliciting ranges of behaviors. The clay model stimulus had a slightly smaller range in dart behaviors than the control assay. Likewise, the video stimulus elicited a higher range of displays, a larger range of charges, and a slightly lower range of darts than the control assay. It is possible that these ''model'' assays do not have the same consistent and positive aggression feedback mechanisms as presented in the MIS or live conspecific assays. Because of this, the most highly aggressive individuals may be the only individuals being provoked to perform aggressive behaviors, and they did so at a decreased rate. This does appear to be the case since the behavioral measures were repeatable at a fairly high rate across all aggression assays (with the exception of charges). Additionally, the assays may have also been less stressful than the constant aggression feedback of the more highly elicited aggression assays. Measuring vasotocin, a

Table 4. Assay and Behavioral Recommendations

		Bites		*Displays*		*Darts*		*Charges*	
		$\bar{x}$	σ^2	$\bar{x}$	σ^2	$\bar{x}$	σ^2	$\bar{x}$	σ^2
Assay 1	Control	Low	Low	Low	Low	Mid	High	None	Low
Assay 2	22.5° mirror	Low	Mid	High	High	High	Mid		High
Assay 3	30 min MIS	High	High	High	Mid	Low	Low		Mid
Assay 4	30 s MIS	High	High	High	High	Low	Low		High
Assay 5	Live conspecific	High	High	High	Mid	Low	Low		Low
Assay 6	Clay model	Low	Low	Low	Low	Mid	Mid		Low
Assay 7	Video stimulus	Low	Low	Low	Mid	Mid	Mid		Mid

Our recommendations for assay usage when the study goal is high mean behavioral expression ($\bar{x}$), and high assay-elicited variance (σ^2). A *dark gray* background is characterized by high expression, an *intermediate gray* background is characterized by moderate expression, and a *white* background is characterized by low expression. The *light gray* column represents no difference in elicited behavior. Note the comparisons presented above are not all statistically significant, but fall upon a spectrum of behavioral expression.

MIS, mirror-image stimulus.

zebrafish homolog of human vasopressin, or serotonin levels might confirm this hypothesis since they have been observed to influence aggression levels in zebrafish.[8,46,47]

The execution of the clay model stimulus assay and video stimulus assays may have also been limited by the technological design of the assay. Other similar constructs could have been implemented that may have altered our observed behavioral response, potentially to closer mimic the MIS and live conspecific assays. For instance, AnyFish is a software package that may be a useful alternative to general video recording because of its highly customizable.[48] This customizable technology would be very useful to determine the primary phenotypic and behavioral causes of an elicited behavioral response in a focal individual through a systematic isolation of traits and presentations, which is much akin to Niko Tinbergen's ''sign stimuli'' idea.[49] Despite the disparate aggression responses induced by the video stimulus in our study, a recent study confirmed zebrafish respond equivalently to a video stimulus as they would a live conspecific.[50] Moreover, a clay model stimulus may elicit a wide range of behavioral responses dependent on the construction quality of the model. Because of these potential shortcomings in our technological design, and the high potential for variability of both clay model and video recording assay designs, we cannot guarantee an expected response as indicated by our data. For direct consistency and standardization, we suggest MIS or live conspecific assays since they are not as highly variable in design.

The inclined MIS assay (assay 2) was different than either of the aforementioned behavioral response assay subsets. This assay was characterized by low bites, high displays, and high darts, while eliciting a mid-level range of bites and darts, and a high range of displays and charges. These behavioral differences may have been attributable to differences in tank size, since the other MIS assays were conducted in a tank that was twice as large as the tank used in the inclined mirror assay. This assay also differed in the type of stimulus that was available because in the inclined MIS assay an individual could completely avoid seeing the mirror, whereas with the flat mirrors the only alternative for a nonaggressive individual was to move as far away as possible. Nevertheless, despite the differences, the assay was conducted according to the existing paradigm,[28] and its behavioral response characterization was unique. If a study intends to elicit a wide behavioral range while maintaining high aggression rates and allowing for an individual to easily escape the potential stressors of a mirror stimulus, we recommend performing the inclined MIS assay.

One of the purposes of this exploratory study was to produce a comparison of commonly used aggression assays, and to provide recommendations for researchers studying aggression. We provide data regarding which aggressive behaviors are expected when quantified using a number of commonly used behavioral assays. For instance, if a goal is to elicit a high number and large range of bites and lateral displays, we would recommend MIS. However, if an aggressive response is desired but without the high feedback stressors we suggest selecting a clay model or video stimulus. A summary of recommendations is presented in Table 4.

New assay paradigms are consistently being adapted and presented to fish aggression literature. For instance, robotic means of eliciting a behavioral response is a novel approach that provides a customizable physical stimulus that can directly interact with the focal organism in three dimensions, rather than a customizable video recording.[51] It has recently been shown that such a biologically inspired robotic zebrafish elicits a species-specific shoaling response.[52,53] Future methodological investigations on other behavioral paradigms are of need as well. For instance, boldness and exploratory behaviors are often quantified and differences between them are subtle, but should be investigated. In the past, similar pursuits have been pursed for other species,[54] which increases the power of the behavioral model. Therefore, we recommend a concise and thorough explanation of all characterized zebrafish behavioral assay paradigms.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Scott P. McRobert, PhD
Department of Biology
Saint Joseph's University
Philadelphia, PA 19131

E-mail: smcrober@sju.edu