Chapter 20

Quantifying Aggressive Behavior in Zebrafish

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Abstract

Aggression is a complex behavior that influences social relationships and can be seen as adaptive or maladaptive depending on the context and intensity of expression. A model organism suitable for genetic dissection of the underlying neural mechanisms of aggressive behavior is still needed. Zebrafish has already proven to be a powerful vertebrate model organism for the study of normal and pathological brain function. Despite the fact that zebrafish is a gregarious species that forms shoals, when allowed to interact in pairs, both males and females express aggressive behavior and establish dominance hierarchies. Here, we describe two protocols that can be used to quantify aggressive behavior in zebrafish, using two different paradigms: (1) staged fights between real opponents and (2) mirror-elicited fights. We also discuss the methodology for the behavior analysis, the expected results for both paradigms, and the advantages and disadvantages of each paradigm in face of the specific goals of the study.

Key words Aggression, Social dominance, Behavior, Ethogram, Event recorder, Zebrafish

1 Introduction

Aggression can be defined as any behavior directed toward another individual with the intention to cause harm [1]. It is usually seen as an adaptive behavior expressed throughout most animals' lives, which has evolved in the context of intraspecific competition for resources, such as food, shelter, mating opportunities, or social status. However, heightened aggression levels may become maladaptive, and in humans they are often associated with psychiatric disorders [2]. Therefore, the study of aggression has been prompted both by fundamental and by applied questions. Despite significant progress in the identification of the neurobiological factors associated with aggression, there is still a need to understand in more detail the neural circuits and the active molecules that control this behavior. Similar to other complex behaviors, aggression is induced by the interplay of genes, neurotransmitters, and hormones, in the building and regulation of neural circuits that appear to be conserved across vertebrate species [3, 4]. Thus, progress in this area needs a model organism with a genetic toolbox

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available that allows for real-time visualization of brain activity and for the precise manipulation of specific neural circuits, in order to enable the mapping of behavior into neural circuits [5].

Zebrafish have already proven to be a powerful animal model for the study of complex cognitive disorders like depression, autism spectrum disorder (ASD), drug abuse, cognitive deficits, and psychoses [6]. Several behavioral paradigms used in rodents to study these disorders have already been successfully developed in zebrafish, such as exploration (open field), anxiety-like (light-dark and alarm substance), locomotion (novel tank), and social and cognitive (shoaling, social preference, predator avoidance, and T-maze) tests [6]. The utility of this species in behavioral neuroscience has grown markedly because of its available molecular (forward and reverse genetic methods [7, 8]), electrophysiological [9], and optogenetic [10] tools, the variety of wild-type lines with distinct behavioral phenotypes [6], conditional transgenic lines [11], and the similarity its genome presents with the human genome, where approximately 70% of the genes have human orthologs [12]. All these features make zebrafish an ideal model for translational neuroscience.

Although zebrafish is a gregarious species that in nature form shoals [13], when allowed to interact in pairs, both males and females express aggressive behavior and establish dominance hierarchies [14–16]. In this species, aggression is commonly used by dominant individuals to get access to spawning sites and protect their social status from competitors [16]. Similarly to other species, the repertoire (i.e., ethogram) of zebrafish agonistic behavior consists of a series of stereotype body postures and movements that have been previously characterized (Table 1) [15]. In dyadic male fights, two distinct phases have been described: (1) A pre-resolution phase, where both fish exhibit the same repertoire of behaviors (display, circle, and bite); this phase lasts until the first chase or flee is observed, which marks the establishment of a behavioral asymmetry between the contestants (i.e., fight resolution); (2) A post-resolution phase, characterized by an asymmetry of expressed behaviors, where all agonistic behaviors are initiated by the dominant fish, whereas the subordinate only displays submissive behaviors. Therefore, the expression of the different aggressive behavior action patterns has a specific temporal structure (Fig. 1). An agonistic interaction usually starts with both opponents exhibiting lateral displays in an antiparallel position and circling each other. Then, it progresses to mutual bites, still in the pre-resolution phase. Finally, in the post-resolution phase, dominant individuals bite, chase, and strike toward subordinates, whereas the latter flee, freeze, and retreat.

Given that fish lack visual self-recognition, when exposed to a mirror, they usually display aggressive behavior toward their mirror image [17]. Therefore, aggressive behavior in fish has been quantified using either their response toward real opponents [14, 15] or toward their own mirror images [17–22]. However, recent studies have questioned

Table 1

Ethogram of zebrafish aggressive behavior (adapted from Oliveira et al. [15])

Behavioral patterns	Description
Displays	In short distance of the opponent, usually less than one body length, fish erects its dorsal and anal fins and flares its body flank toward the opponent
Circle	Two fish approach each other in antiparallel positions with their fins erected and circle one another ascending in the water column. It can last from a few seconds to minutes
Strike	The fish swims rapidly toward the opponent, but no physical contact occurs
Bite	Fish opens and closes its mouth in contact with the body surface of its opponent, usually directed toward the ventral or the posterior parts of the body of the target fish
Chase	Similar to strike but with an active pursuit by the aggressor. This behavior stops when one fish stops chasing and/or the other fish adopts a freeze behavior
Retreat	Fish swims rapidly away from the opponent in response to a strike or a bite
Flee	Continued escape reaction in response to a chase. Fish swims rapidly away from the aggressor
Freeze	Fish stays immobile with all fins retracted and the caudal region downward near the bottom or the surface of the aquaria

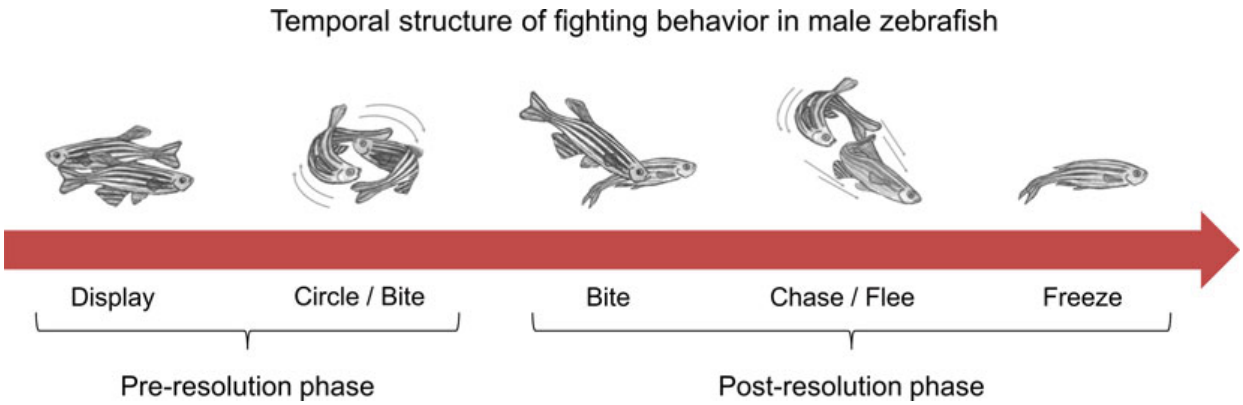

Fig. 1 Zebrafish male fights exhibit a typical temporal structure. Fights can be divided into a pre-resolution phase and a post-resolution phase. The pre-resolution phase is defined by the expression of symmetric behaviors by both contestants, and behaviors such as displays, circles, and mutual bites occur. The post-resolution phase is characterized by a transition to asymmetric expression of behaviors between the opponents, where bites, chases, and strikes are performed by the dominant individual, whereas retreat, flee, and freeze are expressed by the subordinate. The arrow represents the temporal occurrence of each type of behavior in the respective phase (adapted from Oliveira el al. [15])

whether these two tests of aggression are measuring the same aspects of behavior, since they elicit different hormonal responses in cichlid fish [17, 22]. In zebrafish, mirror-elicited fights also failed to arouse the same brain responses as real opponents in gene expression [23] and in the monoaminergic activity [19]. Despite these physiological differences elicited by the two protocols, there are no significant differences between the levels of overt aggression exhibited toward a mirror image or a real opponent [19, 20]. Thus, both protocols seem suitable for quantifying overt aggression measures, but the decision to use one or the other should take into consideration known differences between the two (Table 2), which may be advantageous or disadvantageous, depending on the specific goals of the study. Here, we describe two protocols that can be used to quantify aggressive behavior in zebrafish, using each of these two paradigms: (1) staged fight test, between real opponents, and (2) mirror-elicited aggression test.

2 Materials

1. Electronic balance.
2. Ruler/caliper.
3. Buffered tricaine methanesulfonate (MS222; *see* **Note 1**).
4. Spring scissors.
5. Forceps.

Table 2

Advantages and disadvantages between real-opponent and mirror-elicited fights as tests of aggression in zebrafish

	Real-opponent fight	Mirror-elicited fight
Advantages	– Provide the most natural social stimulus – Promote the establishment of social dominance with the emergence of dominant and subordinate phenotypes	– The opponent's behavior is standardized to that of the focal fish (i.e., it is the same) – Fighting individuals are not exposed to physical injuries, which makes it ethically more acceptable
Disadvantages	– The researcher has no or limited control of the stimulus fish, and the behavior of the focal fish depends to a great extent on the behavior of the opponent – Fighting individuals can be physically injured, and thus it is less acceptable from an ethical perspective	– The fights are unsolved and therefore the focal fish never experiences either a victory or a defeat [21] – Prevents the expression of lateral display in an antiparallel position, which is a common action pattern in real-opponent fights – The dynamics of the fight are atypical, since the opponent never initiates behavior and never displays submissive behavior

6. Fish-holding support (*see* **Note 2**).
7. 27G needle (internal diameter 0.210 mm).
8. Nylon monofilament 0.14 mm.
9. Povidone-iodine (Betadine®) or any other microbicide-like chlorhexidine to disinfect the material.
10. Nail polish.
11. Zebrafish maternity tanks (18 × 10 × 9 cm).
12. Video camera.
13. Multievent recorder software for behavior recording and analysis (Observer XT).

3 Methods

3.1 Animal Housing

The protocols described here were developed using adult wild-type zebrafish of the AB strain (*see* **Note 3**). Fish are kept in a recirculating housing system (ZebTEC Multilinking System, Tecniplast, Italy), at 28 °C with a 14 L:10D photoperiod. The water is monitored for nitrites (<0.2 ppm), nitrates (<50 ppm), and ammonia (0.01–0.1 ppm), and pH and conductivity are maintained at 7 and 700 μSm, respectively. Fish are fed twice a day, except on the day of the experiments.

3.2 Individual Tagging

In staged fights, it is important to identify each individual during the whole interaction, such that the behavior of each opponent can be quantified separately. For this purpose, individuals need to be individually tagged. There are three commonly used procedures to tag zebrafish: fin clipping [15], color tagging with nylon monofilament [24, 25], and color tagging with implanted elastomers [26] (*see* **Note 4**). Here we describe the two methods that are currently used in our lab.

3.2.1 Fin Clips

1. Anesthetize the fish by immersion in tricaine solution (160 mg/L) in a petri dish (*see* **Note 5**).
2. Use the spring scissors to clip the extremities of the caudal, dorsal, or anal fins in different combinations between pairs of opponents.

3.2.2 Color Tagging with Nylon Monofilament

1. Prepare the nylon monofilament by cutting approximately 5 cm; give three or four knots with the help of the forceps in one tip and paint the knots with nail polish (*see* **Note 6**).
2. Cut the other tip of the nylon monofilament in diagonal, in order to be pointed.
3. Place all materials, including the painted nylon monofilament previously prepared, in povidone-iodine (Betadine®) or any other microbicide solution.

4. Anesthetize the fish by immersion in tricaine solution (320 mg/L) in a petri dish.
5. Place the fish in an appropriate bedding (*see* **Note 2**).
6. Insert the hypodermic needle (27G) through the dorsal musculature immediately below the posterior insertion of the dorsal fin.
7. Insert the pointed nylon monofilament already tagged through the needle hole (Fig. 2a).
8. Remove the needle out of the fish body leaving the monofilament behind (Fig. 2b).
9. Give three or four knots, with the help of the forceps, on this tip and paint with nail polish (Fig. 2c, d) (*see* **Note 7**).

3.2.3 Recovery from Anesthesia

1. Fill a zebrafish maternity tank with water (approximately 800 mL) and place the fish to recover after any of the tagging procedures described above. Do not use more than five animals per tank to mitigate stress [27].

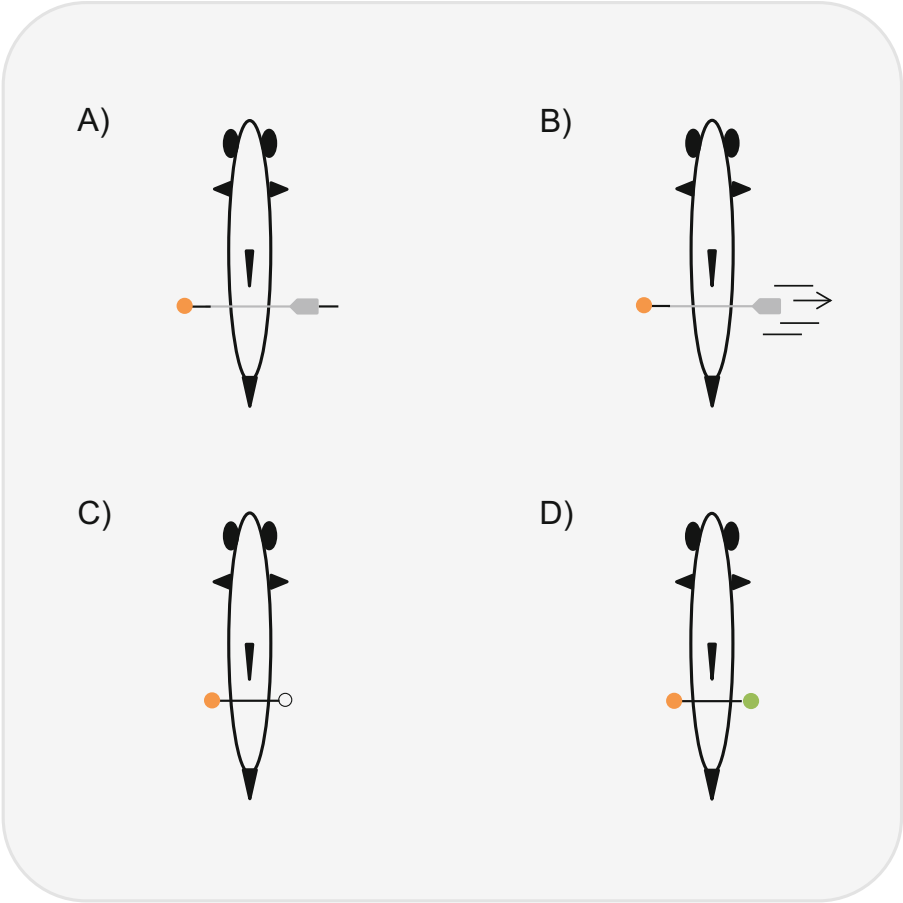

Fig. 2 Color tagging with nylon monofilament. The fish is represented in a top view: (**a**) Insertion of the hypodermic needle through the dorsal musculature of the fish and guiding the nylon monofilament already tagged through the needle hole. (**b**) Removal of the needle leaving the monofilament in place. (**c**) Giving knots on one side of the monofilament. (**d**) Painting it with nail polish (reproduced with permission from Patzner [24])

2. Animals will recover very fast from the anesthesia (in minutes); however, in order to maximize the anesthetic withdrawal, keep animals in the recovery tank for 1 h before moving them back to the home tank (*see* **Note 8**) [28].

3.3 Behavioral Recording

1. We typically use an experimental tank of 32 cm × 20 cm × 15 cm divided into two parts: (1) the posterior part (19.5 cm × 20 cm × 15 cm) containing a mechanical filter and a heater (water temperature is kept at 28 °C also during the tests) and (2) the anterior part (12.5 cm × 20 cm × 15 cm), hereafter designated as arena, where the tests take place (*see* **Note 9**).
2. Cover the back wall of the arena with white PVC, in order to improve contrast between fish and the background in video recordings.
3. Divide the arena into two parts of the same size by a removable PVC partition (Fig. 3): (a) for staged fights, the PVC partition separates the two fish in the right and left sides of the tank (Fig. 3a); (b) in mirror-elicited fights, the PVC partition contains one mirror on each side and is perforated on the sides to allow water flow between the two parts; a second removable partition should be placed in front of it to hide the mirrors from the focal fish before the start of the interaction (Fig. 3b).

3.3.1 Staged Fights

1. Pair the animals according to their weight and standard length (*see* **Note 10**).
2. Prior to the experiment, place each pair in the experimental tank, one fish on each side of the arena divided by the opaque partition, where they stay overnight in visual isolation (*see* **Note 11**, Fig. 3a, b). Before the experiment, set up a standard video camera (*see* **Note 12**) in front of the tank to record the interaction.
3. Gently remove the opaque partition and allow the two fish to interact for a period of 30 min (*see* **Note 13**, Fig. 3a′).
4. At the end of the test period (30 min), a dominant and a subordinate fish should be easily identified by the different behaviors they express (i.e., winners only express aggressive behaviors, and losers only express submissive behaviors); place the partition back into the observation tank to separate the two fish again, and note the identity of the dominant and of the subordinate fish.

3.3.2 Mirror-Elicited Fights

1. Repeat **steps 1** and **2** from the staged fights protocol in Subheading 3.3.1.
2. Gently remove the two opaque partitions that are covering the mirrors, and allow the two fish to interact with each mirror simultaneously (*see* **Note 14**, Fig. 3b′).
3. After the 30 min period, place the two opaque partitions back in place, in order to end the interaction of each fish with its own mirror image.

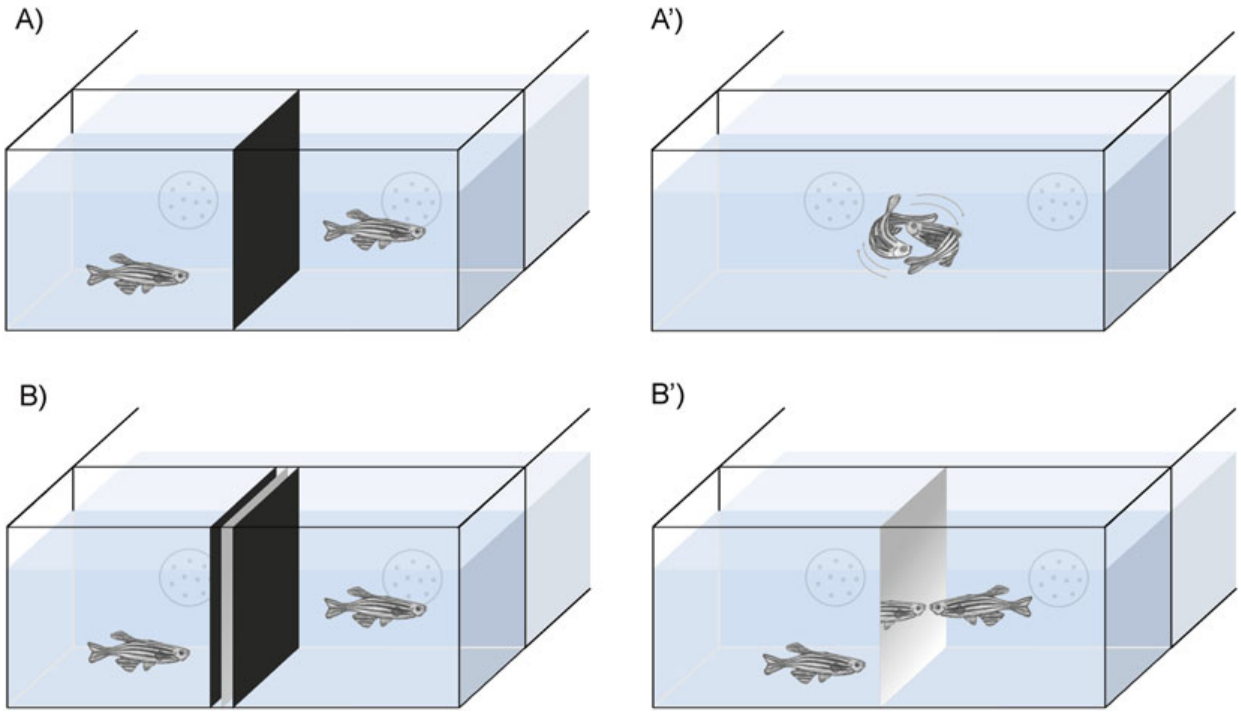

Fig. 3 Observation tanks are divided into a posterior part, which contains a mechanical filter and a heater, and an anterior part where the test takes place (the arena). Perforated plastic circles along the glass dividing the two compartments allow water exchange between the arena and the filter compartments. The arena is divided into two same-size parts by an opaque PVC partition; depending on the test (real-opponent or mirror fight), this partition can be removed or not. (**a**) For real-opponent fights, animals are separated by a removable opaque PVC partition. (**a′**) The opaque divider is removed, and the fish are allowed to interact for 30 min. (**b**) For mirror-elicited fights, the arena is divided by a PVC partition containing one mirror on each side, and a second removable partition is placed in front of each mirror to cover it. (**b′**) The two outer partitions are removed, and the fish are allowed to interact with their own mirror image throughout the test period (30 min)

3.3.3 Quantitative Behavioral Analysis

1. Analyze the video recordings using a computerized multievent recorder (Observer XT, Noldus, Wageningen, The Netherlands).
2. Use the ethogram of zebrafish agonistic behavior to identify the relevant action patterns [15], which are divided into aggressive for dominants (bite, chase, and strike) and submissive for subordinates (freeze and flee).
3. Identify the selected behaviors as states or events, and quantify the frequency or the duration of the respective behaviors (*see* **Note 15**).

3.3.4 Typical Results

For staged fights, a typical encounter starts with mutual displays (lateral displays, circling) characteristic of the pre-resolution phase. In the post-resolution phase when the dominant-subordinate status has already been established, chase and bites are the most frequent action patterns (Fig. 4a).

When comparing staged fights with mirror-elicited fights several differences can be observed (Fig. 4b–d):

1. The latency for the first attack (i.e., bite) is significantly lower in mirror fights when compared to staged fights, which may be a result of mirror "opponents" providing ambiguous information leading mirror fighters to escalate their aggressive behavior faster than individuals fighting a real opponent (*see* **Note 16**).
2. The opposite pattern is observed for the fight resolution time, with staged fights being solved more rapidly (in approximately 7 min) than mirror fights (usually still ongoing at the end of the 30 min observation period). This may result from the fact that during the pre-resolution phase, fish mutually assess their relative fighting ability and adjust their behavior accordingly.
3. Since there is no fight resolution in mirror fights, mirror fighters do not either win or lose the fight; therefore, they do not

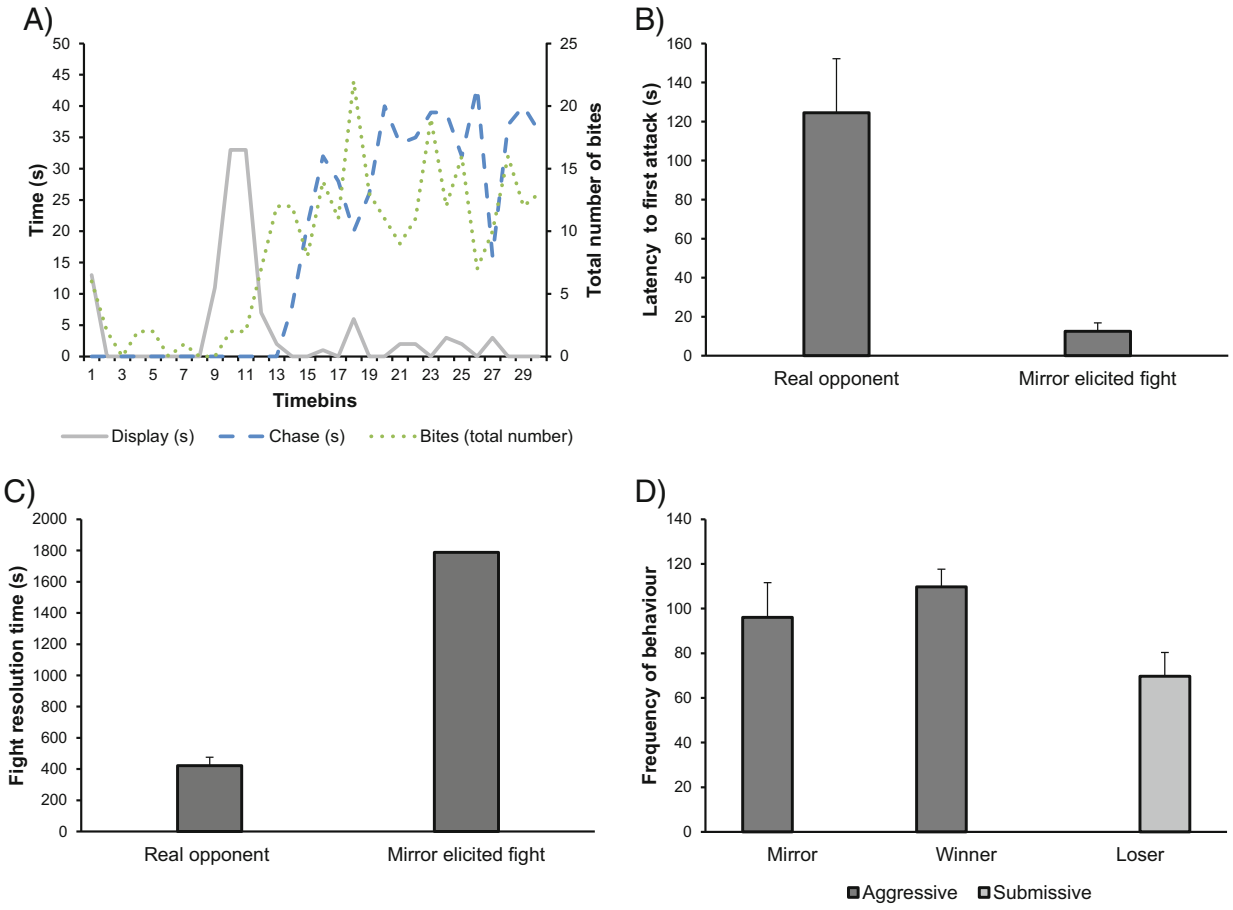

Fig. 4 Typical results for the two protocols used to quantify aggressive behavior. (**a**) Temporal dynamics of a real-opponent fight analyzed in 1 min time bins for the 30 min interaction (unpublished data). The full line represents the time in display, a typical behavior of the pre-resolution phase, and the *dashed* and *dotted lines* represent the time in chase and number of bites, respectively, behaviors typically expressed in the post-resolution phase. (**b**) Mean latencies to the first attack in real-opponent and in mirror-elicited fights (unpublished data). (**c**) Fight resolution time, measured as the time needed for a social hierarchy to be established, in real-opponent and in mirror-elicited fights (unpublished data). (**d**) Mean number of aggressive acts performed in the last 5 min of the 30 min interaction test for winners and losers of real-opponent fights and for mirror fighters; error bars represent the standard error of the mean (reproduced with permission from [19])

adopt the respective dominant or subordinate phenotype, observed in real-opponent fights, despite the expression of significant amounts of aggressive behavior.

4. Indeed, there are no significant differences in the levels of overt aggression between mirror fighters and dominants of real-opponent fights. Thus, one can conclude that a major difference between the two protocols is not so much in the behavior expressed by the focal fish, but rather in the behavior expressed by the opponent.

As a final recommendation, we suggest that researchers intending to use the mirror test to phenotype aggression should first validate it by comparing individual responses between real-opponent and mirror tests. This has been done recently for a set of different cichlid species, and the results appear to be species specific, since in some species (i.e., *Neolamprologus pulcher* and *Astatotilapia burtoni*) the results of the two tests are correlated [18, 29], whereas for other species (i.e., *Telmatochromis vittatus*, *Lepidiolamprologus elongatus*, and *Amatitlania nigrofasciata*), no relationship was found between mirror and real-opponent aggression [29].

4 Notes

1. Buffered tricaine methanesulfonate (stock solution): 4000 mg/L tricaine methanesulfonate (MS222), buffered with tris-base 1 M, pH = 9 to a final pH = 7 solution.
2. The bedding can be a small petri dish filled with aquarium-graded silicone, with a small depression in the middle to hold the fish in a dorsoventral position.
3. One should keep in mind that aggressive behavior might differ between different wild-type strains, as it has been described for other behaviors [30].
4. The choice of the tagging method depends on the experimental procedure to be used. For example, fin clips are normally used for short-term experiments since fin regeneration occurs rapidly, whereas color tagging is more appropriate for long-term experiments, despite being a more intrusive technique. Finally, visible implant elastomers are more suitable for experiments that do not require video analysis because visible implant elastomer tags may be difficult to distinguish in video images (e.g., yellow vs. orange or pink vs. red can be easily confused). Furthermore, color identification may depend on ambient light which becomes a constraint when video recordings are used [26].
5. With this dose of anesthetic a deeper anesthesia will be induced, which promotes a total loss of equilibrium and muscle tone and a very slow ventilation rate (almost absent) [31]. This will

occur very fast. As soon as these signs are present, remove the fish from the anesthetic solution.

6. Beforehand, prepare a sheet with the color combinations that you intend to use to tag the fish, to avoid repetitions of color codes.
7. Leave some clearance between the knots and the fish body to avoid skin infections and interference with body growth.
8. After tagging the animals, there must be a quarantine period before starting the behavioral tests. For fin clips, one should wait at least 24 h and, for color tagging, 10 days to guarantee wound healing. Animals should be monitored during this period for tag loss and health status.
9. The perforated plastic circles along the glass dividing these two parts of the tank allow water exchange between the two compartments (Fig. 3).
10. Since body size is highly correlated with dominance, size differences (length or weight) between opponents should not exceed 10% of total body size, in order to avoid an a priori advantage of the larger individual. Take the opportunity of having fish anesthetized for the tagging procedures to take body measurements (weight, standard length) of all individuals.
11. Previous studies had established different periods of social isolation of 5 days [14, 32] and 24 h [15] as effective to elicit aggressive behavior in zebrafish. However, overnight isolation proved sufficient to induce consistent expression of aggressive behavior for the duration of the tests (30 min) [19].
12. The camera we used had a resolution of 720×576 and frame rate of 25 frames per second; however, higher resolution cameras with higher frame rates are also appropriated.
13. In order to minimize the interaction between the observer and the focal fish, the partitions can be pulled up from a distance with the help of pulleys.
14. Subjects were also tested in pairs in the mirror-elicited test, in order to provide them with conspecific odors, which would otherwise only be present in real-opponent dyads, therefore avoiding confounding effects of putative chemical cues used in agonistic interactions.
15. For behavior quantification, it is important to distinguish between two fundamental types of action patterns, based on the time expression, because this will influence the type of measures that one should take: (1) events are action patterns that are discrete in time (i.e., have very short duration) such that it is difficult to establish their start and finish time (e.g., bites, strikes); the relevant measure of events is their frequency (number of occurrences per unit of time); (2) states are action patterns that have a significant time duration which allows to easily define their start and their

end (e.g., display, chase, freeze, and flee); states can be quantified both in terms of their frequency and their duration (e.g., percentage of time displaying). Latency, defined as the time from some specified time point (e.g., start of the test) to the first occurrence of the relevant action pattern, can also be measured, both for events and for states. Latency to initiate a fight is usually interpreted as a measure of aggressive motivation, whereas frequency and duration of events and states, respectively, reflect the engagement in the interaction. Since the engagement in the fight depends not only on the motivation of the focal fish but also on the response of the opponent, measures of latency are expected to better measure the intrinsic aggressive motivation of individuals. In our protocols, we typically analyze the latency to the first interaction and the frequency and duration of aggressive and submissive behaviors.

16. When laterally displaying to each other, as a way of assessing each other's competitive ability [33], fish can align either in a parallel (head to head) or antiparallel (head to tail) position [34]. However, since there is a left-eye bias in zebrafish for social stimuli, they prefer to display the left side of the body, making the head to tail alignment, which is not present in mirror interactions, more common during mutual displays [20]. Thus, mirror fights also change the structure of the fight making mirror fighters escalate faster than real-opponent fighters.

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