



Nonreversing mirrors elicit behaviour that more accurately predicts performance against live opponents

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Mirror image stimulation has a long history of being used to quantify aggressive behaviour but its suitability has recently been questioned because behavioural responses towards a mirror image and towards a real opponent are not always correlated, and are associated with different physiological responses. These discrepancies might result from lateral-display behaviour, which provides a way for animals, particularly fish, to assess fighting ability during early stages of a contest. With a regular mirror, species that prefer head–tail orientation during lateral display are unable to do so, which might lead to aberrant responses that would not accurately reflect behaviour in a real contest. We designed a non-reversing mirror test by connecting two regular mirrors at a 90-degree angle, allowing animals to see and interact with their image in head–tail postures. We compared behavioural indices in three standardized aggression tests (using a regular mirror, a nonreversing mirror or a size-matched, three-dimensional inanimate model) and in real fights to examine which test best predicted aggression in real fights between mangrove rivulus fish, *Kryptolebias marmoratus*. Individuals tested with both regular and non-reversing mirrors preferred using right-lateral displays, while those tested with a nonreversing mirror delivered more attacks than those tested with the regular mirror and the model. Individuals with higher frequencies of attack towards the nonreversing mirror had higher winning probabilities in real fights. Contests involving individuals that differed considerably in aggression exhibited towards the non-reversing mirror were less intense and shorter in duration. However, individual differences in performance in tests using the regular mirror and model did not predict contest dynamics. These results support the hypothesis that nonreversing mirrors, but not regular mirrors or models, elicit behaviour that corresponds with the fishes' performance during real fights. Our study validated the nonreversing mirror as a new method for quantifying aggression with the potential to broadly impact research ranging from neurobiology and behaviour to population ecology and evolutionary biology.

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Mirror image stimulation is broadly used to evaluate a diverse set of social behaviours in many species including fishes (Archard & Braithwaite, 2011; Campbell, Carter, Wheeler, & Thorgaard, 2015; Clotfelter, O'Hare, McNitt, Carpenter, & Summers, 2007), amphibians (Rudh, Breed, & Qvarnström, 2013), reptiles (Farrell & Wilczynski, 2006), birds (Branch, Kozlovsky, & Pravosudov, 2015; Hirschenhauser, Wittek, Johnston, & Mostl, 2008) and mammals (Taylor, Boutin, Humphries, & McAdam, 2014). Hundreds of studies have utilized this technique to make inferences about individual variation in conspecific-directed aggression (Archard & Braithwaite, 2011; Campbell et al., 2015) and have used behaviour exhibited towards a mirror as an index of aggression (e.g. frequency

of attacks). For example, in studies of Siamese fighting fish, *Betta splendens*, mirror image stimulation has been used to investigate the neurobiological mechanisms underlying aggressive behaviour (Clotfelter et al., 2007; Eisenreich & Szalda-Petree, 2015; Eisenreich, Greene, & Szalda-Petree, 2017). Clotfelter et al. (2007) used a fish's response to its mirror image as an index of aggressive behaviour, and revealed a role for serotonin in modulating conspecific male–male aggression. Aggressive responses to mirror images have also been applied to understanding the architecture of behavioural syndromes (Chang, Li, Earley, & Hsu, 2012; Wilson, de Boer, Arnott, & Grimmer, 2011). Chang et al. (2012) examined the relationships between aggression (readiness to perform opercular display to a mirror image) and both boldness and exploratory tendencies in mangrove rivulus fish, *K. marmoratus*, and discovered that testosterone and cortisol play important roles in mediating correlations between these behavioural traits. Moreover,

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behavioural responses to mirror image stimulation have been employed to test key hypotheses in evolutionary ecology. Taylor et al. (2014) utilized mirror image stimulation to quantify individual differences in behaviour of red squirrels, *Tamiasciurus hudsonicus*, and tested the hypothesis that ecological changes through time drive fluctuating selection and the maintenance of behavioural variation within populations. These studies demonstrate that mirror image stimulation has been used broadly to understand both the proximate and ultimate mechanisms underlying behavioural variation, particularly with respect to aggression.

Quantifying behaviour using mirror image stimulation rather than a real opponent provides substantial advantages because the test reduces variation in behavioural responses due to the opponent's performance (Balzarini, Taborsky, Wanner, Koch, & Frommen, 2014; Kusayama, Bischof, & Watanabe, 2000; Vøllestad & Quinn, 2003), requires fewer study animals (Scherer, Buck, & Schuett, 2016) and avoids possible injuries caused in real fights (Elwood, 1991). Nevertheless, because individuals' behavioural responses (e.g. Ariyomo & Watt, 2013; Arnott, Beattie, & Elwood, 2016; but see Balzarini et al., 2014; Elwood, Stoilova, McDonnell, Earley, & Arnott, 2014) and hormonal responses (Dijkstra, Schaafsma, Hofmann, & Groothuis, 2012; Oliveira, Carneiro, & Canário, 2005) towards a mirror image are not always consistent with the responses towards an actual opponent, the appropriateness of the mirror image stimulation has recently been questioned (Arnott, Ashton, & Elwood, 2011; Cattelan, Lucon-Xiccato, Pilastro, & Griggio, 2017; Earley, Hsu, & Wolf, 2000; Elwood et al., 2014). Moreover, Desjardins and Fernald (2010) also found that, compared with fighting against actual opponents, mirror image stimulation elicits different patterns of immediate early gene expression in the brain. These inconsistencies of behavioural and physiological responses between mirror image stimulation and real fights may be explained by differences in the types of display behaviour that can be utilized (Ariyomo & Watt, 2013; Arnott et al., 2011; Scherer et al., 2016). During a fight, individuals often display one side of their body to present physical traits to an opponent (Jennings, 2012), which might allow animals to assess the size and competitive ability of their opponents (e.g. Jennings & Gammell, 2013) before escalating into high-intensity forms of combat. Lateral display is especially important in fish because it provides an opportunity for individuals to use the lateral line mechanosensory system to assess the opponent without engaging in direct, and potentially damaging, physical contact (Butler & Maruska, 2015). Individuals engage in lateral displays in two basic ways, either with their heads aligned in the same direction (head–head lateral display; Fig. 1a), or in opposite directions (head–tail lateral display; Fig. 1a). An animal may present both sides, or show one side preferentially due to cerebral lateralization (de Santi, Sovrano, Bisazza, & Vallortigara, 2001; Reddon & Balshine, 2010). Regular mirrors only enable the animal to view head–head lateral displays, which is not the norm for many species, including the vast majority of fishes. When exposed to regular mirrors, species that prefer head–tail lateral display are thus unable to acquire information about the opponent in ways that simulate the dynamics of natural contests (Arnott et al., 2011), and this might explain differences between a regular mirror test and a real fight in behavioural performance, hormone responses and patterns of brain activation (Arnott et al., 2011; Desjardins & Fernald, 2010; Oliveira et al., 2005). Another general standardized aggression test is the model test, which allows animals to perform both head–tail and head–head postures by fighting with a size-matched, three-dimensional, inanimate model. Although behaviour exhibited in a model test better predicts aggressive performance in real fights than behaviour exhibited in regular mirror tests (Earley et al., 2000), models elicit considerably lower behavioural responses due to inadequate movement of the model. We therefore present a new

design, the nonreversing mirror test, which can be used to complement regular mirror tests. The nonreversing mirror will be especially useful for animals that typically use head–tail lateral displays during contests because it allows the animals to see a true reflection. We hypothesized that, compared with the regular mirror, nonreversing mirror image stimulation would elicit aggressive behaviour that more accurately predicts performance during real contests. We predicted that animals would initiate aggressive acts towards the nonreversing mirror image earlier than the regular mirror image, because the regular mirror image may elicit fear responses that delay approach behaviour (Desjardins & Fernald, 2010). Moreover, we predicted that individuals would interact for a longer period with the nonreversing mirror image than with the regular mirror image. We also predicted that individuals facing the regular mirror would have a higher frequency of switching between different types of displays than individuals facing the nonreversing mirror because they would struggle to achieve the proper head–tail orientation (Elwood et al., 2014). In real fights, we predicted that contest intensity should decrease (i.e. fewer mutual attacks and shorter contest duration) as differences between opponents in their standardized aggression indices increased. Most importantly, we predicted that aggressive behaviour exhibited during nonreversing mirror tests would best predict behavioural performance and outcome in a real fight.

METHODS

Study Organism

Mangrove rivulus fish (hereafter 'rivulus') inhabit mangrove ecosystems ranging from the Central America to Florida and the Bahamas (Taylor, 2012). This species exhibits highly aggressive behaviour in both the field and laboratory (Hsu & Wolf, 2001; Hsu, Lee, Chen, Yang, & Cheng, 2008; Taylor, 2012). Aggressive responses of rivulus towards mirror images, models and conspecific opponents have been well studied over the past two decades (Earley et al., 2000; Garcia et al., 2016; Hsu et al., 2008) and head–tail lateral displays are preferred in this species (Supplementary Videos S1, S2). All these features make rivulus an ideal study organism in which to validate the nonreversing mirror design. Rivulus is the only self-fertilizing hermaphroditic vertebrate, and exclusive selfing results in completely homozygous genotypes whose offspring are genetically identical to the parent and all siblings (i.e. isogenic lineages) (Avisé & Tatarenkov, 2015). This study used hermaphroditic individuals of 15 isogenic lineages derived from populations throughout the species' expansive geographical range (Appendix, Table A1). All individuals were two generations removed from the wild, and were generated by allowing parents to self-fertilize in the laboratory. Fish were isolated on the day of hatching and kept individually in 1000 ml translucent plastic containers (maintenance container). Every container was filled with 750 ml of 25 ppt synthetic sea water (Instant Ocean[®]) and labelled with a unique code for individual identification. Fish were maintained at ambient temperature (27 ± 1 °C) on a 12:12 h light:dark photoperiod and fed 2 ml of newly hatched brine shrimp (*Artemia*) nauplii every day. The average (\pm SEM) age of experimental animals was 489.60 ± 10.18 days; the average (\pm SEM) standard length of experimental animals was 26.95 ± 0.25 mm.

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The Mechanism of Nonreversing Mirror

In a regular mirror design, the path of light from an individual's right and left sides are reflected once, then perceived by the

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