What do fish make of mirror images?

Julie K. Desjardins* and Russell D. Fernald  
Department of Biology, Stanford University, Stanford, CA, USA  
*Author for correspondence (desjarjk@stanford.edu).

Fish act aggressively towards their mirror image suggesting that they consider it another individual, whereas in some mammals behavioural response to mirrors may be an evidence of self-recognition. Since fish cannot self-recognize, we asked whether they could distinguish between fighting a mirror image and fighting a real fish. We compared molecular, physiological and behavioural responses in each condition and found large differences in brain gene expression levels. Although neither levels of aggressive behaviour nor circulating androgens differed between these conditions, males fighting a mirror image had higher immediate early gene (IEG) expression in brain areas homologous to the amygdala and hippocampus than controls. Since amygdalar responses are associated with fear and fear conditioning in other species, higher levels of brain activation when fighting a mirror suggest fish experience fear in response to fights with a mirror image. Clearly, the fish recognize something unusual about the mirror image and the differential brain response may reflect a cognitive distinction.

Keywords: immediate early genes; hippocampus; amygdala; pre-optic area; egr-1; c-fos

1. INTRODUCTION

What do animals make of seeing their own image in a mirror? Gallup (1968) used mirrors to assess animals’ ability to recognize themselves and based on such tests, most vertebrates do not have self-recognition (e.g. Anderson & Gallup 1999). In fish, observers noted that a mirror image would elicit apparently unconditioned aggressive display (e.g. Betta splendens, Lissmann 1932). Tinbergen (1951) observed that male three-spined sticklebacks displayed aggressive behaviour towards mirror images, suggesting that fish treat mirror images as an intruding individual.

To discover whether fighting an opponent is similar to fighting a mirror image, we measured differences in behavioural, hormonal and brain activity between conditions. We quantified localized differences in immediate early gene (IEG) expression levels as a proxy for neural activation (e.g. Clayton 2000) in context-relevant processing regions of the brain. Based on previous behavioural tests (Burmeister et al. 2005), we hypothesized that IEG measurements might reveal subtle differences between conditions since IEGs are known to play many roles in mediating neural plasticity, including the activation of signal transduction.

Received 16 March 2010  
Accepted 23 April 2010  

* We used qRT-PCR to measure mRNA expression in each region of the brain. Primers for the A. burtoni target genes, egr-1 and c-fos and for control genes, 18s RNA and actin were designed according to published sequences (Burmeister et al. 2005). The qRT-PCR was performed using 30 μl duplicate reactions (SYBR Green, Bio-Rad) and performed on a real-time PCR system (Bio-Rad). Original fluorescence readings were analysed using a curve-fitting real-time PCR algorithm (Zhao & Fernald 2005). Computed cDNA concentrations of the two housekeeping genes (18s and actin) were not significantly different from each other, so we used the geometric mean of these as a normalized standard for each tissue sample. The relative mRNA levels of the target genes (c-fos and egr-1) were calculated as the percentage of the geometric mean of the housekeeping genes.

One-way ANOVAs were used (JMP) to test hormone levels (T, 11KT) and RFPCR data for statistical significance. Tukey’s HSD post hoc tests were conducted to detect pairwise differences between treatments with the overall alpha level at 0.05. A t-test was conducted on total aggression, all components of aggression
differ between males 'mirror' or 'opponent' males (all
components of total aggression between 'opponent' and
opponent (all males who had not been in an aggressive encounter (control, grey bars). Different letters above the bars indicate a significant difference between groups with overall $\alpha = 0.05$.

as well as number of aggressive bouts and aggressive bout time between fish that were aggressive towards a mirror and fish that were not. To test for any relationships between size of the opponent and behaviour, hormone and gene expression, a composite of size difference was calculated as the sum of difference in standard length and difference in body mass. Means for groups and overall test statistics are in appendix A, electronic supplementary material.

3. RESULTS

A. burtoni males fight vigorously to establish and defend territories (Fernald 1977) and will fight with other males when separated by a clear barrier (e.g. Burmeister et al. 2005). In our experiments, there was no difference in total aggression or the components of total aggression between 'opponent' and 'mirror' males (all $t < 0.992$ and all $p > 0.34$; appendix A, electronic supplementary material). Among individuals that fought an opponent, there were no statistical relationships between total aggression and any of the components of total aggression or size of the opponent (all $p > 0.15$; appendix B, electronic supplementary material). While androgens did not differ between males 'mirror' or 'opponent' males ($T$: $p = 0.59$; $11$KT$: p = 0.157$) animals in these groups had higher androgen levels than control males (figure 1, $T$: $p = 0.002$; $11$KT$: p = 0.006$).

Surprisingly, mirror and regular fights had strikingly different effects on the brain. 'Mirror' males had higher levels of egr-1 expression in the homologue of the hippocampus, DI, than 'opponent' males or controls ($p = 0.008$). By contrast, c-fos expression in DI was significantly higher in 'opponent' males, when compared with 'mirror' and control males ($p = 0.006$). Males who fought a mirror image had much higher egr-1 and c-fos expression in Dm than males who fought an opponent or controls ($egr$-1: $p = 0.03$; c-fos: $p = 0.02$). In the Cce, there were no differences in egr-1 or c-fos expression among any of the males (figure 2: egr-1: $p = 0.252$; c-fos: $p = 0.14$). In the POA, egr-1 and c-fos males who fought a mirror or an opponent had higher expression than controls ($egr$-1: $p = 0.02$; c-fos: $p = 0.05$), however, there was no difference between the fighting males (mirror and opponent, egr-1: $p = 0.295$; c-fos: $p = 0.203$). Opponent size did not influence egr-1 or c-fos expression in any brain areas (all $p > 0.21$; appendix B, electronic supplementary material).

4. DISCUSSION

Males fighting an opponent through a clear barrier or fighting their mirror image showed similar behaviour, circulating androgens and similar gene expression in the POA and the Cce but vastly different gene expression in DI (hippocampus) and Dm (amygdala) Both of these nuclei receive multimodal sensory inputs (Northcutt 2006). When males were aggressive towards their mirror images, egr-1 mRNA was higher in DI, while both egr-1 and c-fos mRNA was higher in Dm. Increases in both c-fos and egr-1 reflect higher immediate and long-term neural activity possibly including an increase in the transcription of late-acting genes and neuronal firing in the amygdala, as suggested by egr-1 and c-fos. In DI, the mirror possibly elicits an increase only in transcription of later acting genes associated only with egr-1. Interestingly, c-fos expression was higher in DI of males who had interacted with a true opponent. The significant differences in brain activity show that males recognize and respond to something about the mirror, but what?

Perhaps the responses reflect fear associated with a mirror image. In the hippocampus (DI), egr-1 may be acting as a transcription factor for later-acting genes coding for stress responses including mineralocorticoid, glucocorticoid and NMDA ligands and receptors (Bannerman et al. 1995). In mice, learning a spatial task associated with mild stress activates physical activity-related genes differentially in the hippocampus (Cavallaro et al. 2002). It seems plausible that increased egr-1 activity in DI may signal the encoding of stress-related spatial information in animals fighting their mirror image. However, the increased c-fos expression in DI, exclusively in the males who had interacted with a true opponent does not support this hypothesis. An alternative hypothesis is that the mirror image represents a perfectly size-matched opponent. Theoretical models and behavioural evidence in a number of taxa suggest that aggressive behaviours between territory holders increase with decreasing size difference between combatants (e.g. Maynard Smith & Price 1973). If true, we would expect an inverse relationship between opponent size difference, aggression and IEG expression in the amygdala, which we did not. Fear induces associative long-term potentiation in the amygdala (Rogan et al. 1997), a nucleus that contains a large population of corticotropin-releasing hormone containing neurons (Schulkin et al. 1997) known to be active when animals face a fearful stimulus.

The mirror image presentation may induce fear in A. burtoni males because it is a completely novel stimulus, not interpretable based on past experience because the mirror ‘opponent’ does not react in familiar ways.
Alternatively, the opponent and the mirror conditions may be seen as two different stimuli resulting in two different brain gene expression patterns and neither induces fear. However, since the central difference between these conditions is in the amygdalar response, this probably reflects fear in these animals.

The differential increase of IEGs in the homologues of the hippocampus and amygdala of fish fighting their own image shows that IEGs can provide important information not found in behavioural responses or hormone levels. These brain activity measurements show that the animal considers fighting a mirror image different from fighting a conspecific suggesting that these fish may have cognitive capacities that go beyond Tinbergen’s (1951) suggestion that they were limited to fixed action patterns. While using mirrors to stimulate and test cognitive abilities in vertebrates is widespread, caution should be used when interpreting a response towards a mirror as identical to that towards a conspecific opponent.

We thank H. Baier, K. Maruska, R. Carpenter, J. Fitzpatrick, B. Grone and 3 anonymous reviewers for comments on this MS, H. Cooper and K. Eaton for technical help. J.K.D. was supported by an NSERC PDF and R.D.F. by a Jacob Javits Award from NIH NS034 950.

All experimental procedures have been approved by Stanford University and APLAC (IACUC assurance number: A3213–01).


