



Feature binding in zebrafish

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The binding problem is the brain's fundamental challenge for advanced sensory processing: objects in the outside world possess multiple features, which must be bound into a cohesive perceptual representation. Although there is suggestive evidence that nonmammalian vertebrates (and possibly insects) may support it, this rudimentary form of sensory syntax is ascribed primarily to cortex or similarly complex avian structures. The experiments reported here provide evidence that a small vertebrate lacking cortex supports visual feature binding for social behaviour. Zebrafish, *Danio rerio*, displayed spontaneous preference for images of other zebrafish in which the visual attributes of form and motion were paired in a meaningful fashion, while each attribute in isolation was rendered ineffective as a cue for discrimination. The ability to conjoin the two features was robust and remarkably flexible. These results challenge the notion that feature binding may require cortical structures and demonstrate that the nervous system of small vertebrates can afford unexpectedly complex computations.

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The earliest stage of biological image processing is widely regarded as a highly specialized process supported by detectors selectively tuned to individual features of the incoming stimulus, such as orientation, colour and motion (Zeki & Shipp 1988); these different attributes, initially encoded within distinct neural structures, must be reassembled into a unified perceptual representation of the outside world (Treisman 1996). It has been recognized for several decades that this more advanced stage of processing is highly demanding and can fail under some circumstances (Wolfe & Cave 1999), thus representing a challenging 'binding' problem for sensory systems (Roskies 1999).

Current attempts to relate existing theories of feature binding (Treisman 1996) to known neural structures rely primarily on cortex (Zeki & Shipp 1988; Shafritz et al. 2002; Robertson 2003; Botly & De Rosa 2009). The preferential attribution of feature-binding capabilities to this highly evolved mammalian structure is motivated by the lack of conclusive evidence that perceptual feature binding may be performed by animals with allegedly more limited neural resources than mammals (chapter 3 in Shettleworth 2008). Birds (which lack cortex) possess this ability (Cook 1992; Blough & Blough 1997; Katz et al. 2010) but their brains are equipped with neural structures of equivalent estimated potential to those of mammals (Jarvis et al. 2005).

The above statements specifically refer to perceptual feature binding: the ability to carry out perceptual discriminations that require access to a bound sensory representation and cannot be performed by relying on individual features alone (see General Discussion for further clarification). At present there is no conclusive experimental evidence for this ability in reptiles, amphibians or fish (there is also no definitive evidence from invertebrates such as insects, even though these animals have been shown to display remarkably complex visually guided behaviour; Collett & Collett 2002; Srinivasan 2010).

Because nonhuman primates find conjunction tasks especially difficult (Smith et al. 2004), it is conceivable that creatures such as fish may not support this ability at all, particularly in view of the current notion that binding is intimately linked to higher-level cognitive phenomena such as attention (Treisman 1996; Robertson 2003). On the other hand, there is substantial suggestive evidence from other forms of binding-like operations that non-mammalian vertebrates (e.g. toads, *Bufo bufo*; Ewert et al. 1979) and some insects (e.g. honeybees, *Apis mellifera*; Schubert et al. 2002) may support this type of cognitive operation; furthermore, fish possess neural structures that may be homologous with the mammalian cortex (Mueller & Wullmann 2009). The question remains open.

In this study, I investigated whether the zebrafish, *Danio rerio*, a small teleost, supports feature binding and whether it relies on this ability for the purpose of social aggregation (Miller & Gerlai 2011: 'shoaling'). I used stimuli specifically designed to exclude the possibility that the results may be explained by the animal

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relying on a single visual feature (Shepard et al. 1961; Smith et al. 2004), requiring instead compulsory conjunction of form and motion. These two visual attributes are widely believed to be processed by different cortical regions in primates (Zeki & Shipp 1988) encompassing a rich circuitry which, by some morphological accounts, may appear orders of magnitude more complex and articulate than the zebrafish brain.

METHODS

Animals and Test Apparatus

I used wild-type zebrafish (age range 4–12 months) bred and maintained by trained staff in a dedicated facility (Institute of Medical Sciences, Aberdeen, U.K.). Outside testing, fish were kept inside a 10-litre storage tank (average density two fish per litre) attached to a recirculated system (Aquatic Habitats, Apopka, FL, U.S.A.) at 27 °C on a 14:10 h light:dark photoperiod and never exposed to heterospecifics. They were fed brine shrimp twice a day (at 0930 and 1630 hours). During testing, one fish was transferred to a test tank measuring 25 × 13 cm and 11 cm high; water within the test tank came from the storage tank and room temperature was thermostatically controlled. The two furthest sides of the test tank were placed against two identical LCD monitors (Samsung EX1920W) while the remaining sides were lined with nonreflective white paper. The two monitors were clones controlled by one computer but the two regions of the monitors that were adjacent to the tank were different, allowing independent control over the images displayed to the two sides. All stimuli were generated and presented using custom Matlab (Mathworks, Natick, MA, U.S.A.) software; the operating system (linux) simultaneously controlled a webcam located above the test tank (44 cm from the water surface) and acquired images of 320 × 240 pixels at 4 Hz (see [Supplementary Movie S1](#)). These images were stored on the hard drive for automated offline analysis (see below). To tailor image quality to the tracking algorithm, as well as to avoid the fish inspecting irrelevant features lying above the tank, the sides of the test tank were raised 24 cm above the water surface using black nonreflective cardboard and indirect lighting was generated by a halogen lamp. Each fish was tested only once for a given experimental condition and stimulus generation/data acquisition were automatically controlled by computer software; after placing the fish in the test tank and launching the software, I would leave the room and return at the end of the experiment to repeat the process for a different fish. After testing, fish were returned to the breeding stock. Ethical approval for all the research reported in this study was obtained from the University of Aberdeen Ethical Review Committee. The work was deemed as nonregulated by the Home Office Inspector; however, input was received from the Home Office Inspector and the Named Veterinary Surgeon and the care of all fish was under the remit of the Animals (Scientific Procedures) Act 1986. No animal licence was required because the behavioural procedures used here were harmless and only involved wild-type animals.

Visual Stimuli and Presentation Protocol

The footage shown in [Supplementary Movie S2](#) was obtained by filming wild-type zebrafish from the same colony that comprised the test fish. In addition, synthetic movies ([Supplementary Movies S3–S5](#)) were generated by adding small images of a zebrafish, a manipulated zebrafish or a needlefish, *Xenentodon*, to a grey background. I refer to these images as 'icons' and illustrate the procedure for the movie shown in [Supplementary Movie S3](#); identical procedures were adopted for the other movies. Individual icons were initially placed within the image at random spatial locations and

made to drift horizontally at a constant speed of 6.5 cm/s. Half the icons faced left and half faced right; half moved to the left and half to the right. Icons that were facing left (right) were also moving left (right) in the congruent condition; the opposite pairing was adopted for the incongruent condition (this was simply obtained by playing the movie backwards). When two icons overlapped within the image, the icon added more recently was painted over the other icon (partial occlusion, see [Supplementary Movie S3](#)). All movies lasted 16 s and were generated using a cyclical structure: the end of the movie matched the beginning of the movie, so that the movie could be played smoothly for many repetitions without glitches. The footage clip was similarly selected so that the first and last images were almost identical (see [Supplementary Movie S2](#)), resulting in a smooth transition during repetition (no detectable glitch). Each phase (test/baseline) lasted 8 min (30 movie cycles). The movie presented on one end of the tank was 8 s out of phase with the movie presented on the other end; this means that even during baseline phases, when the same movie was presented on both ends, two different portions of the movie were presented at a given time. When different movies were presented on the two ends (test phase), the movie presented on a given monitor was alternated between monitors from fish to fish to eliminate potential lateral bias (all data were realigned to the same notional side for analysis and presentation purposes). Any such bias would also be factored out by subtracting the baseline phase from the test phase ([Figs 1–4](#)); however, in practice there was no significant bias during the baseline phase ([Supplementary Fig. S1](#)). I retained this phase in all experiments for two reasons: (1) it enabled me to confirm, on an experiment-by-experiment basis, that the apparatus and procedures were correctly calibrated (i.e. unbiased); (2) it allowed the fish to acclimatize and recover from the stress of being caught. The baseline phase displayed the original movie for experiments shown in [Fig. 1](#), the congruent stimulus for experiments shown in [Fig. 2](#) except the yellow/magenta symbols (see figure legend) and blank screens for experiments shown in [Fig. 4](#). For experiments shown in [Fig. 3](#), the baseline phase displayed the congruent zebrafish stimulus for experiments involving zebrafish stimuli (black/yellow/red symbols) and the congruent needlefish stimulus for experiments involving needlefish stimuli (blue/magenta symbols); for the form-only experiments in which both zebrafish and needlefish stimuli were presented during the test phase (green symbols in [Fig. 3b](#)), the baseline phase displayed blank screens.

Movie Tracking

I wrote software specifically tailored to the images collected during the experiments; the algorithm was therefore robust and efficient in the absence of any human intervention (see [Supplementary Movie S1](#)). The software relied on standard subtraction methods for motion detection (McIvor 2000): the average image was computed across all 16 min of movie recording (baseline plus test phases) and subtracted from each individual frame. The software then applied a threshold of 6× the standard deviation of intensity values within each frame and performed cluster analysis of the threshold image around the location of minimum intensity (fish image was dark). The resulting cluster was selected (red-tinted pixels in [Supplementary Movie S1](#)) and its centroid coordinates were used as position pointers for the test animal (yellow cross in [Supplementary Movie S1](#)). After the animal's position had been identified on every frame, the software automatically selected (via edge detection) an active area for the test tank (indicated by blue rectangle in [Supplementary Movie S1](#)) and rescaled all longitudinal positions to range between 0 and 1 within this region (so that 0.5 corresponded to equidistance from the two monitors). In [Fig. 1](#) the active area is indicated by the outer rectangle and the individual tracked positions by dots.

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