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RESEARCH ARTICLE

Disruptive colouration in reef fish: does matching the background reduce predation risk?

Genevieve A. C. Phillips[1],*, Martin J. How[2], Julia E. Lange[3], N. Justin Marshall[1] and Karen L. Cheney[3]

ABSTRACT

Animals use disruptive colouration to prevent detection or recognition by potential predators or prey. Highly contrasting elements within colour patterns, including vertical or horizontal bars, are thought to be effective at distracting attention away from body form and reducing detection likelihood. However, it is unclear whether such patterns need to be a good match to the spatial characteristics of the background to gain cryptic benefits. We tested this hypothesis using the iconic vertically barred humbug damselfish, *Dascyllus aruanus* (Linneaus 1758), a small reef fish that lives among the finger-like projections of branching coral colonies. Using behavioural experiments, we demonstrated that the spatial frequency of the humbug pattern does not need to exactly match the spatial frequency of the coral background to reduce the likelihood of being attacked by two typical reef fish predators: slingjaw wrasse, *Epibulus insidiator* (Pallas 1770), and coral trout, *Plectropomus leopardus* (Lacépède 1802). Indeed, backgrounds with a slightly higher spatial frequency than the humbug body pattern provided more protection from predation than well-matched backgrounds. These results were consistent for both predator species, despite differences in their mode of foraging and visual acuity, which was measured using anatomical techniques. We also showed that a slight mismatch in the orientation of the vertical bars did not increase the chances of detection. However, the likelihood of attack did increase significantly when the bars were perpendicular to the background. Our results provide evidence that fish camouflage is more complex than it initially appears, with likely many factors influencing the detection likelihood of prey by relevant predators.

KEY WORDS: Visual ecology, Predator–prey relationships, Fourier analysis, Animal behaviour, Disruptive camouflage

INTRODUCTION

Animals use visual camouflage to avoid detection and/or recognition by predators and prey (Cott, 1940; Thayer, 1909) by using different strategies, such as background matching, masquerade, countershading and disruptive colouration (defined in Stevens and Merilaita, 2009a). Background matching requires that the body colour (hue), brightness (luminosity) and/or pattern elements closely resemble that of a specific background (in specialist camouflage) or a number of backgrounds [in generalist camouflage (Stevens and Merilaita, 2009a; Stevens et al., 2011)].

For example, the body colouration of the green tree frog, *Agalychnis callidryas*, has a similar spectral reflectance to the leaves of the tree on which they rest (Emerson et al., 1990) whereas the giant cuttlefish, *Sepia apama*, is able to change body patterns to match a wide range of backgrounds (Zylinski et al., 2011). Background matching is most effective when animals are relatively stationary, as movement will often break camouflage and cause an animal to become more detectable to predatory visual systems (Hall et al., 2013; Ioannou and Krause, 2009; Julesz, 1971). Countershading is used to describe the difference in brightness between the ventral (lighter) and dorsal (darker) sides of a fish. Lighter ventral shading is hypothesised to match the luminosity of the bright sun-lit background when viewed from below, and *vice versa* for the dorsal side (Stevens and Merilaita, 2009a).

Disruptive colouration uses highly contrasting pattern elements that occur near the edge of the animal or across the body to break up the body outline, interrupting normal object recognition pathways so the animal's form is no longer recognisable (Cott, 1940; Cuthill and Székely, 2009; Stevens and Cuthill, 2006; Stevens and Merilaita, 2009b; Stevens et al., 2009; Thayer, 1909). In Cott's (1940) pioneering work on animal colouration, he suggested that the sub-principle of maximum disruptive contrast (in terms of colour or luminance) between adjacent pattern elements was one of the most effective mechanisms for distracting attention away from a focal animal. Cott (1940) used the black and white, vertically barred humbug damselfish *Dascyllus aranus* as one of the main examples to illustrate this tenet. However, disruptive colouration should also have some resemblance to the background against which it is viewed, in terms of colour, pattern and luminance (Fraser et al., 2007). For example, Kelman et al. (2007) demonstrated that the degree of luminance contrast in disruptive markings displayed by cuttlefish did not exceed the luminance contrast in the experimental backgrounds. Additionally, when the luminance contrast between the pattern and background is similar, moths with disruptive edge markings have higher survival rates compared with those with non-disruptive elements, suggesting that disruptive colouration rarely acts in isolation (Stevens et al., 2006). Matching the background luminance is important in disruptive colouration; however, whether disruptive markings additionally have to match the background in terms of spatial scale to prevent detection has not been specifically tested to our knowledge. This is despite evidence that there are significant differences in the spatial frequency of conspicuous and cryptic animal body patterns (Cheney et al., 2014; Godfrey et al., 1987). Interestingly, Cott (1940) did not make any predictions about the spatial characteristics of optimal disruptive colouration in animal body patterns.

In this study, we used humbug damselfish to examine whether the spatial frequency of disruptive pattern elements needs to closely match the spatial frequency of coral backgrounds to provide the fish benefit from a reduction in the likelihood of attack. While there may be other functions to the black and white pattern of the humbug,

[1]Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia. [2]School of Biological Sciences, The University of Bristol, Bristol BS8 1TQ, UK. [3]School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia.

*Author for correspondence (gacphillips@gmail.com)

G.A.C.P., 0000-0002-2347-2321

List of symbols and abbreviations

1D FFT	1D fast Fourier transformation
D/T	double/twin cone photoreceptors
FFT	fast Fourier transformation
RGC	retinal ganglion cell
SL	standard length
SRP	spatial resolving power (cycles deg^{-1})
θ	minimum resolvable angle (deg)

such as communication, confusion or 'dazzle', these are not specifically investigated in this study. As outlined in Fig. 1A, fish with a similar pattern to the background, whether that background is plain (Fig. 1Ai) or patterned (Fig. 1Aiii), are more likely to be cryptic from the perspective of a predator, compared with if a pattern is highly contrasting with the background (Fig. 1Aii). To understand the design and success of various camouflage strategies, we must consider how colour patterns are viewed by relevant signal receivers (Endler, 1983). Therefore, we first measured the visual acuity of two reef fish predators using information on the anatomy of their eyes and the density of photoreceptors in the area of the eye most likely used for focussing a clear image (Collin and Pettigrew, 1989; Ullmann et al., 2012). This information was combined to apply relevant blurring to images used in behavioural assays and natural scenes, so that they represented a predator's-eye-view of a scene. Next, we used behavioural experiments with the same two predatory fish species to investigate whether there is a reduction in the likelihood of attack for humbugs when viewed against backgrounds of similar and mismatched spatial frequencies (number of within-pattern elements), measured using fast Fourier transform (FFT) analysis [similar to previous methods (Cortesi et al., 2015b)]. Finally, we assessed field images from the Great Barrier Reef to quantify the spatial frequency of humbug damselfish against natural coral backgrounds. We discuss the implications of our findings in relation to disruptive contrast strategies in both marine and terrestrial predator–prey relationships.

MATERIALS AND METHODS
Study species
The three-barred humbug damselfish, *D. aruanus* (hereafter referred to as a humbug), forms close associations with branching scleractinian coral heads with vertical finger-like protrusions (Allen et al., 2003; Randall and Allen, 1977; Randall et al., 1997). Humbugs are diurnally active, and rarely move more than 1 m from their home coral head, preferring to hide within coral branches when predators approach (McCormick and Weaver, 2012; Sale, 1970, 1971). The two predators in this study were chosen for their different hunting strategies: slingjaw wrasse (*Epibulus insidiator*) are slow-moving, short-range predators (over a distance of a few cm) whereas coral trout (*Plectropomus leopardus*) are ambush predators that approach their prey rapidly, attacking from a few metres away (Schott et al., 2014). Predators were caught from the waters surrounding Lizard Island, Great Barrier Reef, Australia (14°41′06″ S, 145°26′32″ E) using barrier and hand nets (slingjaw wrasse, N=6) or hook and line (coral trout, N=9). These two species are also relatively easy to house and train in aquaria (Cortesi et al., 2015a; Vail et al., 2013, 2014), and have been shown to feed on humbugs in the wild (John et al., 2001; St John, 1999).

Predator spatial acuity
We calculated the spatial acuity of both predators to estimate how they would potentially perceive humbug patterns against experimental and natural backgrounds. We used retinal whole-mounts from five fish of each species to measure the highest density of photoreceptors in their retinas as this would represent the highest possible visual acuity before any summation can occur in the retinal ganglion cell (RGC) layer or beyond (Land and Nilsson, 2012). Retinal whole-mounts were generated by removing the retina from the eye, fixing the tissue and then mounting the entire retina on a slide with the photoreceptors pointing towards the viewer. Photoreceptors were then counted using specialised software on a modified microscope, using design-based stereology methods similar to those previously published elsewhere (Coimbra et al., 2012; de Busserolles et al., 2014a; Ullmann et al., 2012) and described in detail in the Appendix.

Spatial resolution was calculated both as the minimum resolvable angle (θ, in deg) by the eye and in the number of cells subtended by 1 deg of visual arc [spatial resolving power (SRP) in cycles deg^{-1}]. θ can then be used to calculate the smallest detectable size of an object at a given distance. In comparison, the SRP provides us with the reciprocal information, in terms of how many cycles (i.e. black and white lines) could be discriminated in 1 deg of visual arc (Land and Nilsson, 2012).

Calculations of θ followed previously published methods (Land and Nilsson, 2012), where the finest grating an eye can resolve has an angular period of 2Δϕ, where Δϕ is the inter-receptor angle and is calculated as follows:

$$\Delta\phi = s/f, \tag{1}$$

where *s* is the distance between photoreceptor centres and *f* is the focal length or posterior nodal distance=2.55×lens radius, where 2.55 is Matthiessen's ratio. Matthiessen's ratio is the ration between the focal length and lens radius, and ranges from 2.2 to 2.8 in marine and freshwater fish species where it has been empirically measured (Matthiessen, 1882).

Only cone cells were used for calculating minimum separation, and we assumed that all cone cells contribute to the visual task. In both fish, cone cells were significantly larger than rods, and are in fact the only cells reliably visible (using the inner segment), probably due to the predominantly diurnal ecology of both fish. It has been suggested that only double/twin (D/T) cones within fish visual systems convey spatial information, similar to chickens (Osorio et al., 1999), probably due to their high abundance. However, as the function of D/T cones is still unknown, and single cones are also probably involved in spatial discrimination [particularly in the high-density regions of the retina – see Siebeck et al. (2014)], we have used both D/T and single cones in all acuity calculations. Minimum separation was measured using the mean number of cells in 1 mm in the densest region of cone cells in the retina (Table 1). Our results represent the highest possible visual acuity, before any summation can occur in the ganglion cell layer, or beyond.

Behavioural experiments
A series of paired-choice experiments were used to test the hypothesis that humbugs benefit from a reduction in the likelihood of attack when their body patterns exactly match the spatial frequency of their coral background. Predators were housed in individual aquaria (slingjaw wrasse: 1.2×1.2×0.4 m; coral trout: 2.2×1.2×0.4 m) in the flow-through seawater system at Lizard Island Research Station, Australia, and were fed either pilchards or prawn twice per day during the acclimation period. Each aquarium was divided into two arenas using black corrugated plastic board (thickness=6 mm) as a partition. A door was cut into the middle of
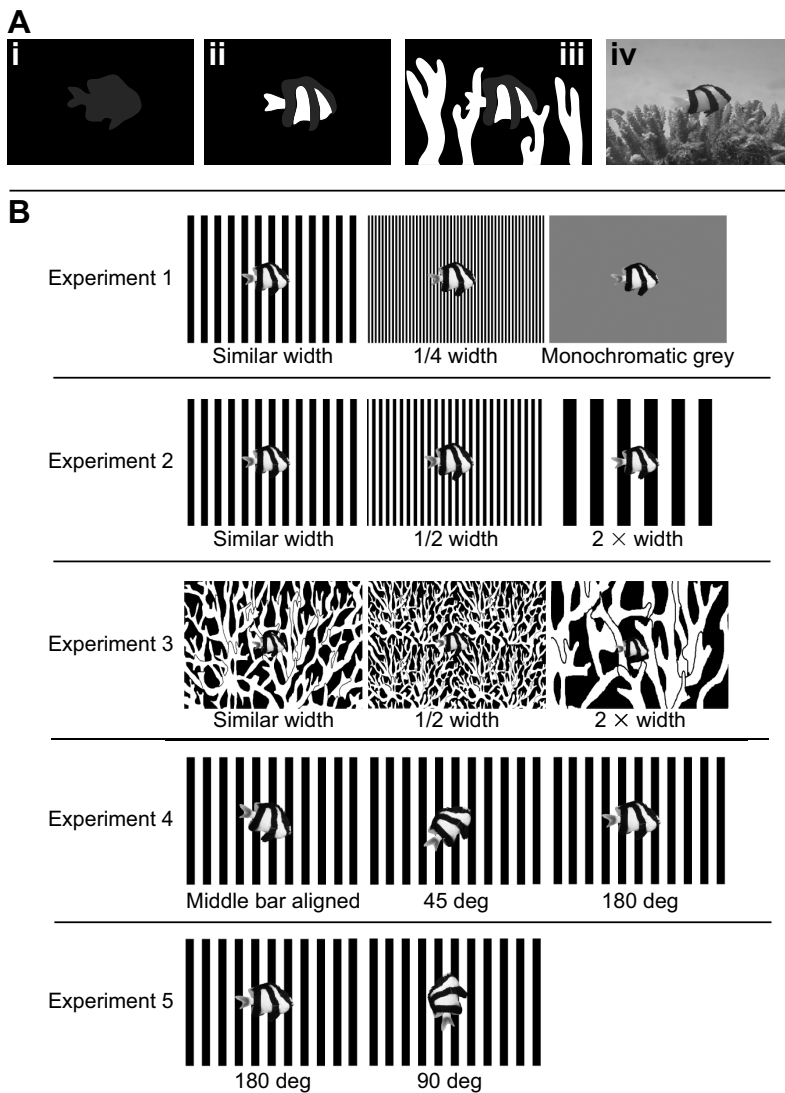
Fig. 1. The humbug's barred body pattern may have evolved to increase crypsis from the perspective of potential predators. (Ai) An unpatterned fish against a low spatial frequency background has good background-matching camouflage; (Aii) humbug damselfish against a low spatial frequency background is highly conspicuous; (Aiii) humbug damselfish against a coral background (similar spatial frequency) would have a good match to the background; and (Aiv) humbug damselfish on a reef, swimming near the branching coral head that it resides in (photograph by G.A.C.P.) (figure modified from Cott, 1940). (B) Backgrounds used in predation experiments. 'Width' refers to the width of the bars or coral branches in the background relative to the middle bar of the humbug damselfish. In Experiments 4 and 5, the angle below the backgrounds refers to the angle by which the fish is rotated.

the partition to allow fish access into the experimental arena (Fig. 2). Tanks were screened off with shade cloth to minimise external disturbances. A submersible video camera (Hero2, GoPro, San Mateo, CA, USA) was placed on a stainless-steel tripod in the middle of the aquarium to film the experiment. The tripod did not interfere with the fish entering the test arena and after a period of acclimation they were not distracted by the camera and tripod.

Behavioural experiments took place over five separate periods of 8–12 weeks from January 2012 to March 2014. In each trial, individual predators were required to search for humbugs that were placed against experimental backgrounds, which varied in spatial frequency. With slingjaw wrasse, we were able to use a laminated cut-out photograph of a humbug placed against each background, which the slingjaw wrasse approached and attacked to receive a food reward from above. However, we were unable to train coral trout to perform the experiment without real prey items; therefore, we used euthanised humbug individuals.

Experimental backgrounds were designed in Adobe Illustrator (CS5.1, Adobe Systems Incorporated, Mountain View, CA, USA). To reduce any response bias that could be caused by hue or saturation contrast, all backgrounds were designed and constructed using black, white or monochromatic grey (50%) with the total

number of pixels in the image altered to a 50:50 ratio of black:white. All backgrounds were printed using only black ink on a Deskjet Printer (HP470, Hewlett-Packard, Palo Alto, CA, USA) and laminated prior to testing. We did not test whether lamination affected the reflectance of the objects or backgrounds as most experimental objects were made using the same materials, thus standardising the glare amongst the backgrounds and objects.

We tested the hypothesis that experimental backgrounds with the same spatial frequency as the vertical bars of humbugs would provide the most protection from predation (Fig. 1B). The spatial frequency of backgrounds varied slightly for each of the two predator species, as slingjaw wrasse are likely to target juveniles and sub-adults whereas coral trout tend to predate on larger adult humbug damselfish (St John, 1999). The laminated photograph of a humbug used in slingjaw wrasse experiments was 30 mm (total length). The width of the middle bar in the pattern of this humbug was 4 mm. We used multiple copies of the same humbug photograph. Humbugs used in coral trout experiments were all adults [determined by a lack of blue pigmentation on the ventral fins; size range: standard length (SL)=26–63 mm], and the width of their middle bar was approximately 8 mm. The humbug image in the slingjaw wrasse experiments was representative of a juvenile fish.

Table 1. Counting parameters and minimum resolvable angles (θ) for individual retinas (*Epibulus insidiator*, *N*=8; *Plectropomus leopardus*, *N*=7)

n	L/R	Counting frame (μm)	Sampling grid size (μm²)	No. sites	PCD (cells×10³ mm⁻²)	Mean PCD (cells cm⁻¹)	D (mm)	α (deg)	SCoE	θ (deg)	Acuity (cycles deg⁻¹)
E. insidiator											
1	L	100×100	565,468	298	84.7	2569	4.28	10.38	0.068	0.092	12±0.88
1	R	50×50	553,680	300	33.6*		4.28	10.38	0.074		
2	L	50×50	549,900	304	68.0		4.10	10.83	0.076		
2	R	50×50	580,608	296	104.8		4.10	10.83	0.056		
3	L	50×50	454,840	301	55.6*		3.34	13.22	0.082		
3	R	50×50	414,735	304	88.4		3.34	13.22	0.062		
4	L	50×50	502,645	351	63.2		4.00	11.09	0.039		
P. leopardus											
1	L	100×100	1,466,980	299	11.4	1222	7.75	5.78	0.054	0.103	10±0.24
2	L	100×100	846,240	423	15.0		8.00	5.60	0.040		
2	R	100×100	846,240	505	12.5		8.00	5.60	0.056		
3	L	100×100	1,276,390	302	16.2		6.56	6.82	0.053		
3	R	100×100	1,260,870	299	18.7		6.56	6.82	0.067		
4	R	100×100	1,456,010	298	16.1		7.25	6.17	0.054		
5	R	100×100	880,722	350	15.2		6.68	6.70	0.064		

Mean θ was calculated using one retina from each individual (i.e. *N*=4 for *E. insidiator*). For some retinas, counts were not possible over sufficient areas due to remaining pigmented epithelium obscuring large areas of the retina (i.e. *E. insidiator* individual 1, right eye), which in turn produced approximate or potentially inaccurate values for peak cell densities. These were therefore not included in the means and have been marked in the table with an asterisk (*). Mean peak cone cell density (PCD) is noted in cells cm⁻¹ for clarity in calculating θ, which needs an estimate for the number of cells in 1 cm. *n*=individual number; L/R, left or right eye; D, lens diameter; α, angle subtending 1 mm on the retina; SCoE, Schaeffer's coefficient of error.

Predators were tested in five separate behavioural experiments in which the prey and the background varied in terms of spatial frequency (Fig. 1B). Backgrounds were either regular black and white vertical bars (Experiments 1 and 2) or stylised natural coral head backgrounds generated from photographs (Experiment 3). The width of the bars (or coral branches) was determined using an 8 mm width as 'similar' to that of the humbug body pattern (based on measurements from wild-caught fish). We then designed backgrounds with '1/4 width', '1/2 width' and 2×width of the 8 mm humbug body pattern. For the slingjaw wrasse backgrounds,

we used 4 mm as the middle bar width (in the humbug body pattern) and so the experimental backgrounds had their bar width adjusted accordingly (Fig. 1). We also tested the hypothesis that humbugs would more likely be attacked when the orientation contrast between the humbug body pattern (based on the middle bar) and the background was high (Experiments 4 and 5).

Training

Predators were first trained to associate an A4 laminated paper target mounted on a Perspex board with food. This training was carried out differently for each predator species due to differences in their behaviour and physiology (Randall et al., 1997). Both species of predator were trained to enter an experimental arena and eat a piece of prawn (slingjaw wrasse) or pilchard (coral trout) attached via a clear fishing line to a plain-white laminated paper background. Once slingjaw wrasse were able to approach and eat food from a background, they were trained using positively reinforced operant conditioning to attack a laminated image of a plain black humbug and given a food reward in the middle of the arena from the experimenter. Images of humbugs and the humbug shape used in training were attached to the backgrounds using double-sided Velcro®. Once coral trout entered the arena to eat the pilchard within 60 s consistently, they moved onto the testing phase, where the pilchard was replaced with a euthanised humbug. Humbugs were euthanised in a seawater solution of 0.2 ml of clove oil per litre of seawater (according to ethics approval QBI/192/13/ARC). Prior to each trial, humbugs were rinsed thoroughly to remove any traces of clove oil and were attached using colourless fishing line to the background.

Testing

A distractor background (with no humbug fish) was present for each trial to ensure that predators searched for humbugs on a background, rather than striking backgrounds at random. We pseudo-randomised the end of the tank in which the backgrounds were placed (left or right), the location of each background (left, right, centre) and the spatial frequency of the distractor background (no humbug attached) to prevent the predator associating a particular location or background with food. Fish blood (2–5 ml) from defrosted,
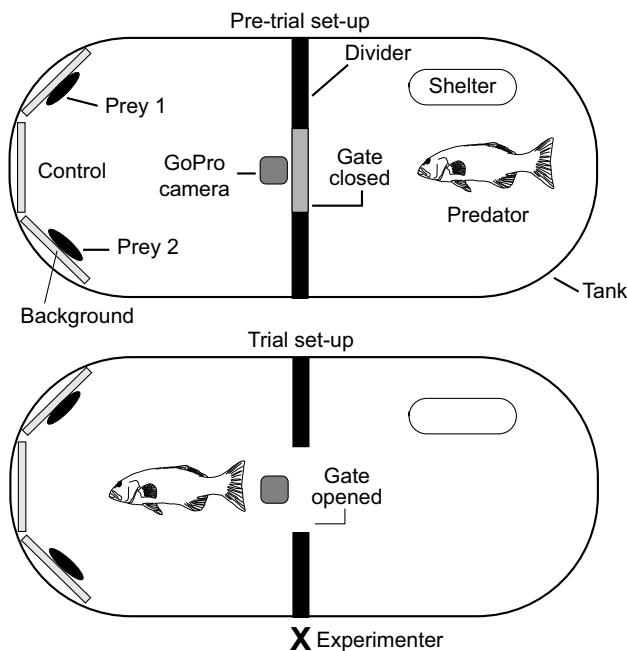

Fig. 2. The experimental set-up used in behavioural choice tests showing the position of the visual backgrounds, prey items and the experimenter. The top diagram shows the pre-trial set-up, with the predator excluded from the testing arena. The bottom diagram shows the dividing gate removed and the predator in the experimental arena.

commercially available pilchards was added to the experimental arena in front of all three backgrounds to reduce olfactory cues from individual humbugs and to motivate predators to attack humbugs. The water was agitated to distribute the blood and to ensure that it did not interfere with the predator's ability to see the background or humbugs.

Trials started when the door was opened and the predator could enter the experimental arena, and ended when the predator attacked a humbug. If the predator took longer than four minutes to attack the humbug, this indicated a lack of motivation to feed and the trial was terminated. In all trials, the humbug and background combinations, time of day of the trial (am or pm), SL of the humbugs (coral trout only) and the location of the chosen background were recorded. A total of six slingjaw wrasse were used (Experiment 1: $N=6$; Experiment 2: $N=6$; Experiment 3: $N=5$), one of which failed to complete Experiment 3 (Table S1). A total of nine coral trout were used throughout the study (Experiment 1: $N=5$; Experiment 2: $N=8$; Experiment 3: $N=6$), all of which completed at least one experiment, with three fish completing all three experiments (Table S1). All predators were presented with a minimum of six repeats of each background combination in each experiment (total trials completed by each predator in individual experiments: minimum $N=14$; maximum $N=30$). Three predators did not complete all background combinations presented to them: two refused to complete one trial each (BMJ12 and BJF12) whereas one refused to complete four trials (DJF13; Table S1). Experiments were conducted in early morning and early evening for the coral trout and during daylight hours of 10:00 h and 15:00 h for the slingjaw wrasse to simulate their respective crepuscular and diurnal predation behaviours. Additionally, experiments that were trialled during the winter months (May–June) were less successful than those in the summer months (January–March) as predators were more motivated to attack prey during these months, presumably due to an increase in metabolism (and therefore hunger) with the increased summer water temperatures. Individual predators were used multiple times in the experiment (each predator completed several trials). The randomisation of each background location and the end of the tank that the experiments were completed ensured a control for any learning bias within consecutive trials.

Statistical analyses

The likelihood of attack for humbugs against particular backgrounds was analysed using a modified Bradley–Terry generalised mixed-effects model (Bradley and Terry, 1952) with a binomial response (background attacked or not attacked). To take into account individual variability between predators and the repetition of tests on each individual, fish identity was added as a random factor within the model. Time of day (am, pm), end of tank in which the backgrounds were placed (left or right), the distractor background used, the location of each of the test backgrounds (left, right and centre) and the trial number were also included as fixed factors; however, these were insignificant (all $Z>0.26$ and all $P\geq0.07$) and were removed from the final model. In coral trout experiments, predators did not choose prey based on body size alone: the size of prey that were attacked was not significantly different to the size of prey that was not attacked (Experiment 1, size range=34–55 mm, paired $t_{84}=-0.98$, $P=0.33$; Experiment 2, size range=26–57 mm, $t_{167}=0.46$, $P=0.65$; Experiment 3, size range=32–63 mm, $t_{107}=-0.70$, $P=0.48$). Therefore, we did not consider prey size further in our analyses. The results of the Bradley–Terry model were then fitted to a logistical function (plogis) to determine the probability of the humbug being chosen

(based on background). Analyses were performed in R 3.1.3 [2015-03-09, 'Smooth Sidewalk' (R Development Core Team; https://www.r-project.org)] using the glm, t.test and lmer functions [MASS package (Venables and Ripley, 2002)], kruskal.test (stats package) and the posthoc.kruskal.nemenyi.test functions [PMCMR package (Pohlert, 2015)]. Data fitted the assumptions of the models.

Image analyses

We then quantified how well humbug body patterns matched the spatial frequency of experimental and natural backgrounds. To do this, we used Fourier analysis techniques to quantify the frequency characteristics of the humbug and the different experimental backgrounds that had been blurred to reflect predator visual acuity. First, bitmap images of the experimental backgrounds with humbugs were created in Adobe Photoshop (Adobe Photoshop CS5.1) and saved as 2048×2048 pixel bitmap images (.bmp). These images were then manipulated using previously published methods (Caves et al., 2016) to approximate predator visual acuity. In brief, the FFT of a single channel of the image was multiplied by a modulation transfer function with a contrast of <2% at the minimum resolvable spatial frequency (i.e. smallest detail). This value was chosen as it represents the minimum contrast threshold for fish under bright light conditions (Douglas and Hawryshyn, 1990). Full images were then recovered using a reverse Fourier transform, resulting in an image where spatial information spanning angles <θ were not present. The following parameters were used to blur the image: distance from the viewer to the image (initially set at 100 cm as this approximates the viewing distance in behavioural trials), the width of the image (estimated using the length of the humbug, set at 5.5 cm total length, which was based on an average adult humbug body length), and the θ of the predator in degrees. Luminance was standardised in these images as they only contained black and white pixels at a ratio of 50:50. We also took some images of humbugs against the coral in their natural environment to identify how they would appear to predators at different distances. We used the same manipulation techniques as described above. The luminance was not standardised as these were natural images; however, we only analysed the green channel of the images as this reflects the best channel for contrast sensitivity in fish eyes.

To analyse the spatial frequency contrast between the vertically barred humbug and the background, we applied a 1D fast Fourier transformation (1D FFT) to the blurred images in a custom-designed MATLAB script (R2014a, MathWorks, Natick, MA, USA). The frequency of 10 same-length horizontal transects (at 0 deg) through the body of the humbug were averaged to calculate the peak spatial frequency of the vertical bars, which was then compared with the mean of 10 randomly positioned background transects of the same length and orientation (see Fig. S1; Figs 3 and 4). If the peak frequency of the humbug bars is similar to that of the background, it suggests that the two objects contain similar frequencies along the horizontal plane (i.e. at 0 deg or 180 deg). The results were plotted showing the peak frequency of the humbug and the background, and also showing the difference between the peak frequency of the fish and the background.

RESULTS
Predator spatial acuity
Both predators had distinct differences in photoreceptor density across the retina, with the highest concentrations of cone cells generally falling within the central region. The highest density of cone cells in the slingjaw wrasse was 2569 photoreceptors cm^{-1} compared with 1222 photoreceptors cm^{-1} in the coral trout
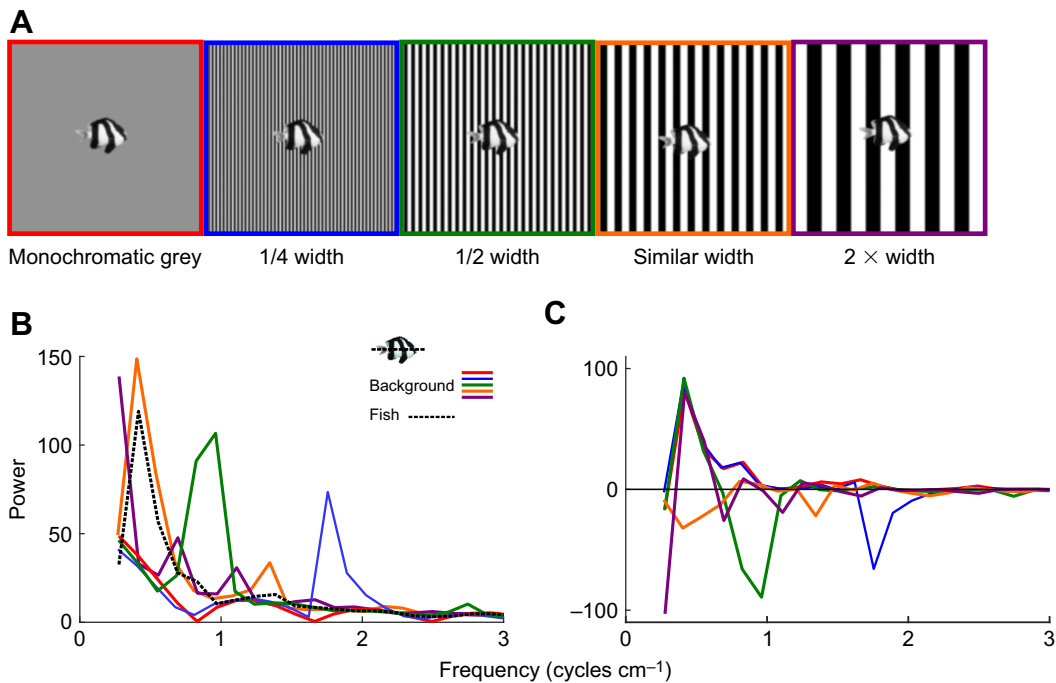
Fig. 3. A series of 1D fast Fourier transform analyses along a horizontal plane for the humbug damselfish and the background using images of the experimental set-up. All images were blurred using a minimum resolvable angle of 0.1 deg and are viewed at a distance of 100 cm. (A) The blurred experimental images indicating the similarity between the spatial frequency of the humbug and the different experimental backgrounds. (B) The peak frequency of the background (coloured lines) and the humbug (black broken line) in cycles cm^{-1}. (C) The difference between the peak frequency of the background and the fish. An identical spatial frequency between the fish and the background would produce overlapping lines in B and a horizontal line at y=0 in C. A higher peak frequency in the fish results in a positive peak on the y-axis in C.

(Table 1). The highest visual acuity calculated with photoreceptor counts agrees with previously published calculations using ganglion cell densities (*Choerodon albigena*, blue-tusk fish: 2880 ganglion cells cm^{-1}; *P. leopardus*: 1225 ganglion cells cm^{-1}: Collin, 1989, 2008). Therefore, we assume no convergence from photoreceptor to ganglion cell in these retinal regions at least, and that photoreceptor densities provide a good estimate of acuity for tasks involving small objects. θ was calculated as 0.092 deg and 0.103 deg for the slingjaw wrasse and coral trout, respectively, based on the number of cone cells in the highest density regions (Table 1). These spatial acuities correspond to minimum resolvable gratings of 1.74 mm and 1.45 mm at a viewing distance of 100 cm, respectively. To simplify the subsequent

analysis, a θ of 0.10 deg was used to approximate both predators' visual resolution.

Image analysis

All eight backgrounds used in behavioural experiments were blurred to a θ of 0.10 deg to determine how predators would perceive the difference between background and humbug pattern. Fig. 3 shows that the humbug peak frequency was most similar to the 'similar width' barred background (orange lines) whereas the other barred backgrounds have distinctly different peak frequencies. Unlike the humbug pattern, there is no one distinctive peak frequency in the 'natural' coral backgrounds (Fig. 4), which has peaks both lower and higher in spatial frequency than that of the humbug. When the
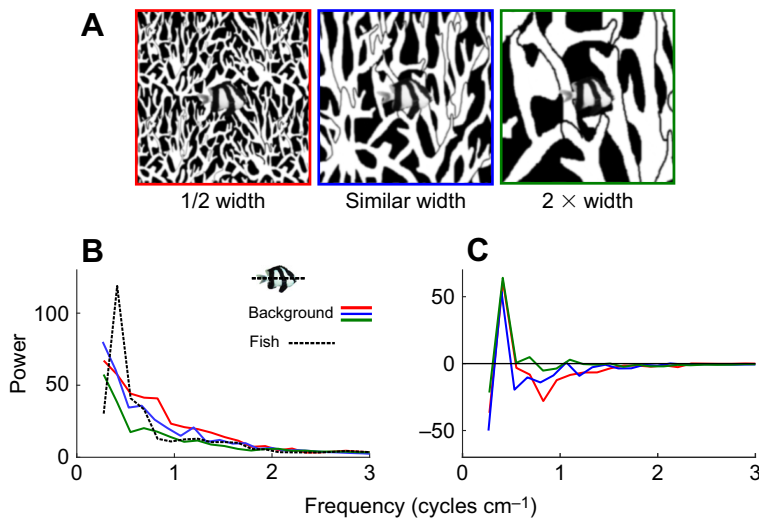

Fig. 4. A series of 1D fast Fourier transform analyses along a horizontal plane for the humbug damselfish and the natural backgrounds used in behavioural experiments. (A) Similarity between the spatial frequency of the humbug and the different natural backgrounds. (B) Horizontal spatial frequency. (C) Difference between fish and background. (See Fig. 3 for conventions.)
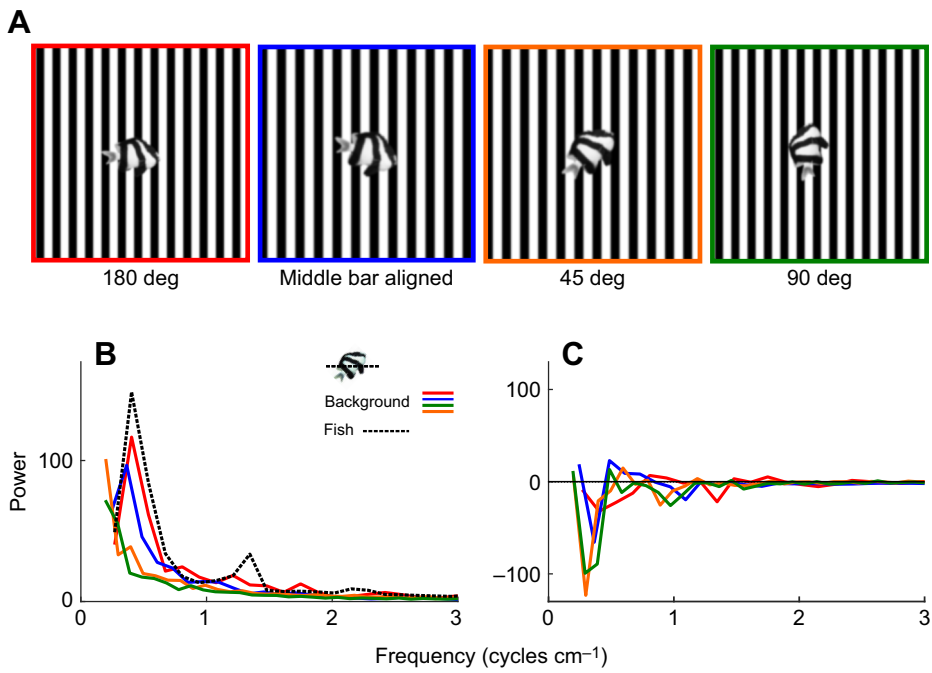
Fig. 5. Fast Fourier transform analyses of the humbug damselfish and the background when the humbug is rotated through different angles. These backgrounds were used in the behavioural trials testing whether angle contrast with the background affected the likelihood of predation. (A) Effect of fish orientation on the spatial frequency contrast. (B) Horizontal spatial frequency. (C) Difference between fish and background. (See Fig. 3 for conventions.)

orientation of the humbug was analysed, it was clear that fish angle influences the peak frequency contrast between the humbug and the background in the horizontal plane, with the greatest differences seen when the fish was at 45 deg or 90 deg to the background (Fig. 5).

We also blurred an image of humbugs against a coral head from the field (humbugs in the wild) and analysed it using the FFT analysis. Only the green channel of the image was analysed to prevent additional colour information influencing the spatial frequency analysis. At close range, the peak frequencies of the humbug body pattern were very different from the coral background (Fig. 6), perhaps due to the wider range of natural frequencies in the background, possibly corresponding to the regular, bold patterning of the humbug, compared with the irregular and less-bold patterning of the branching coral head (branching coral heads lose the structure of their branching when flattened in a 2D image). This image was then blurred to represent increasing viewing distances beyond 100 cm from both a human and predator's perspective, and to
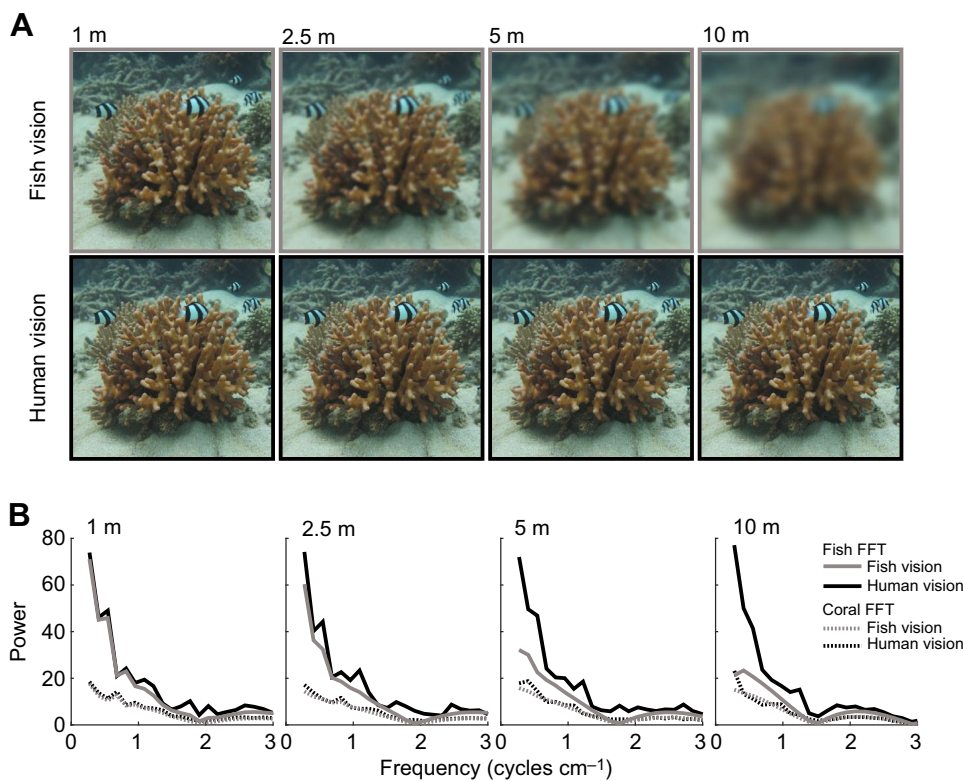

Fig. 6. A comparison of natural images of humbug damselfish against their natural background using a blurring algorithm, and subsequent fast Fourier transformation analyses. (A) Natural images of humbug damselfish above a coral head viewed over four different distances by a fish predator (top row: minimum resolvable angle=0.1 deg) and a human for comparison [bottom row: minimum resolvable angle=0.007 deg (Land and Nilsson, 2012)]. The water is modelled as clear, shallow water and the images do not take into account the differing spectral sensitivities of the predator (or human). (B) The graphs show the respective 1D fast Fourier transformation (FFT) peak frequency in cycles cm^{-1} for the humbug and the background for both viewers (i.e. both fish and human predators).

identify if, at a particular distance, the humbug pattern more closely matched that of the background (Fig. 6). Indeed, by a distance of 500 cm, the humbug body statistics do more closely match that of the coral background from a fish predator's perspective but not from a human's perspective.

Behavioural experiments

The likelihood of humbugs being attacked varied depending on the spatial frequency of the background. In Experiment 1 (grey, similar width and ¼ width bars), humbugs were least likely to be attacked when viewed against a background with a similar spatial frequency to its own body pattern (Fig. 7A; slingjaw wrasse: grey versus similar width: $Z=2.16$, $N=6$, d.f. residuals=15, $P<0.05$; ¼ width versus similar width: $Z=2.75$, $N=6$, d.f. residuals=15,

$P<0.01$; coral trout: grey versus similar width: $Z=5.66$, $N=5$, d.f. residuals=12, $P<0.001$; ¼ width versus similar width: $Z=3.48$, $N=5$, d.f. residuals=12, $P<0.001$), with the grey background providing the least protection from both predators. When viewed by the slingjaw wrasse, there was no statistical difference in the likelihood of attack for the humbug when viewed against the ¼ width background compared with the monochromatic grey (Fig. 7A; slingjaw wrasse: grey versus ¼ width: $Z=0.405$, $N=6$, d.f. residuals=15, $P=0.69$).

In Experiment 2 (½ width, similar width, 2×width bars), humbugs were again least likely to be attacked when viewed against a background with a similar spatial frequency and, interestingly, when also viewed against a slightly higher spatial frequency to their body pattern (Fig. 7B; slingjaw wrasse: ½ width
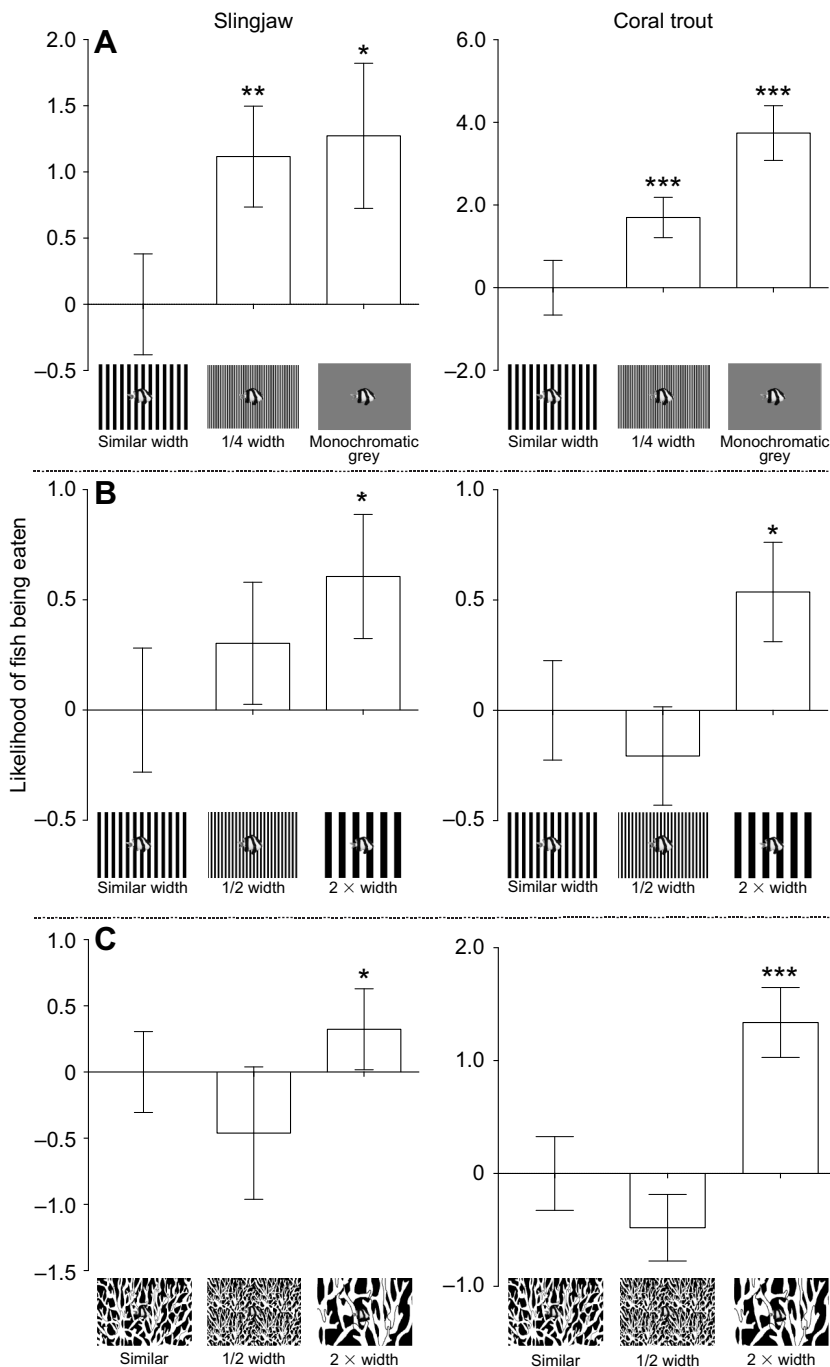

Fig. 7. Results of the behavioural trials using predatory fish in Experiments 1–3. (A) Experiment 1 (slingjaw wrasse, $N=6$; coral trout, $N=5$), (B) Experiment 2 (slingjaw wrasse, $N=6$; coral trout, $N=8$), and (C) Experiment 3 (slingjaw wrasse, $N=6$; coral trout, $N=6$) for both predators. The likelihood of being chosen against a particular background was compared with the background with the least contrast to the humbug body pattern (the null background; predation likelihood set at 0.5). Likelihoods >0.5 suggest prey is more likely to be chosen compared with the null background whereas likelihoods <0.5 suggest prey is less likely to be chosen. Significant differences in probability are marked by an asterisk: *$P<0.05$; **$P<0.01$; ***$P<0.001$. Error bars represent logistical s.e.m. All comparisons are between the null background (similar spatial frequency) and the test background. Further results from additional pairwise comparisons are presented in more detail in the Results section.

versus similar width: $Z=1.10$, $N=6$, d.f. residuals=15, $P=0.28$; 2×width versus similar width: $Z=2.16$, $N=6$, d.f. residuals=15, $P<0.05$; coral trout: ½ width versus similar width: $Z=-0.93$, $N=9$, d.f. residuals=23, $P=0.35$; 2×width versus similar width: $Z=2.38$, $N=9$, d.f. residuals=23, $P<0.05$). The lowest frequency background provided the least protection, consistent with results from Experiment 1.

In Experiment 3, when stylised coral backgrounds were used, there was a decreased likelihood of being attacked when viewed against the similar width or ½ width branching corals. There was no significant difference in the protection afforded by the coral that was most similar in terms of spatial frequency to the humbug body pattern, and that of the smallest branching coral (Fig. 7C; slingjaw wrasse: ½ width versus similar width branches: $Z=-1.50$, $N=5$, d.f. residuals=12, $P=0.134$; coral trout: ½ width versus similar width branches: $Z=-1.63$, $N=6$, d.f. residuals=15, $P=0.103$). When viewed by the slingjaw wrasse, there was no difference in the risk of attack between the similar width and 2×width branching corals (Fig. 7C; slingjaw wrasse: 2×width versus similar width branches: $Z=1.06$, $N=5$, d.f. residuals=12, $P=0.29$). In fact, in slingjaw wrasse behavioural trials, the only time humbug stimuli showed reduced likelihood of attack against natural backgrounds was when the ½ width branching coral was paired with the 2×width branching coral. In this case, there was a significant reduction in attack likelihood if viewed against the ½ width branching coral (Fig. 7C; slingjaw wrasse: 2×width versus ½ width branches: $Z=-2.49$, $N=5$, d.f. residuals=12, $P<0.05$). For the coral trout predator, humbugs gained significantly more protection when viewed against the coral with similar spatial frequency to their own body pattern compared with the widest branching corals (Fig. 7C; coral trout: 2×width versus similar width branches: $Z=1.34$, $N=6$, $P<0.001$).

In Experiments 4 and 5, there was a significant increase in the likelihood of being attacked when the angle of the humbug was at 90 deg to the background (Fig. 8B; slingjaw wrasse: 90 deg versus 180 deg: $Z=2.081$, d.f. residuals=2, $N=4$, $P<0.05$; coral trout: 90 deg versus 180 deg: $Z=2.938$, $N=4$, d.f. residuals=14, $P<0.01$). In all other angle contrast scenarios, the likelihood of attack did not significantly change with angle contrast, although for both species there was a non-significant trend for decreased attack likelihood (Fig. 8A; slingjaw wrasse: stripe matched versus 45 deg: $Z=-1.807$, $N=5$, d.f. residuals=16, $P=0.071$; stripe matched versus 180 deg: $Z=-0.513$, $N=5$, d.f. residuals=16, $P=0.608$; 45 deg versus 180 deg: $Z=0.383$, $N=5$, d.f. residuals=16, $P=0.702$; coral trout: stripe matched versus 45 deg: $Z=-1.317$, $N=4$, d.f. residuals=13, $P=0.188$; stripe matched versus 180 deg: $Z=-1.615$, $N=4$, d.f. residuals=13, $P=0.106$; 45 deg versus 180 deg: $Z=-0.932$, $N=4$, d.f. residuals=13, $P=0.351$).

DISCUSSION
Summary
Our results demonstrate that highly contrasting pattern elements used in disruptive colouration do not have to exactly match the spatial characteristics of the background to reduce the likelihood of attack by potential predators. Interestingly, backgrounds with a slightly higher spatial frequency provided humbugs with a reduction in the likelihood of attack, presumably due to predators being unable to detect the prey items against these backgrounds compared with other backgrounds. Our results are consistent when considering the orientation of the humbug against the background, as the likelihood of attack only increased when the orientation contrast was maximised (humbug at 90 deg to the background). The results for both behavioural testing and image analysis were similar
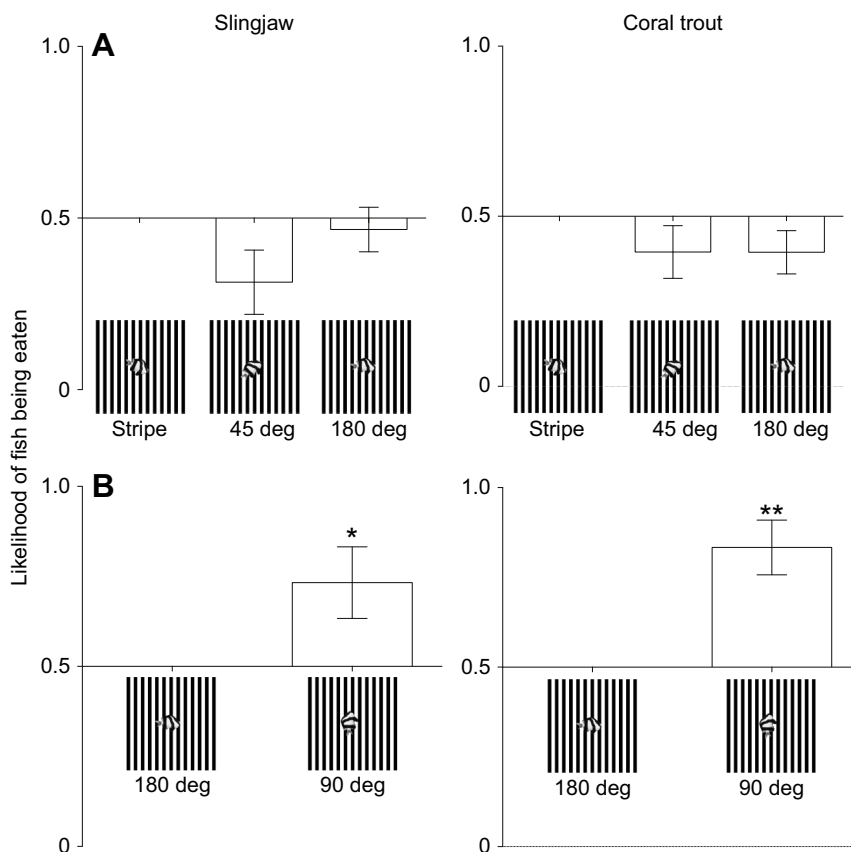

Fig. 8. Results of the behavioural trials using predatory fish in Experiments 4 and 5. (A) Experiment 4 (slingjaw wrasse, $N=5$; coral trout, $N=4$), and (B) Experiment 5 (slingjaw wrasse, $N=5$; coral trout, $N=4$). (See Fig. 7 for conventions.)

between the two predator species, despite differences between their visual systems and hunting strategies, suggesting that the humbug body pattern has evolved to be effective against a wide range of vertebrate visual systems.

Background matching and disruptive colouration

Predator behavioural trials suggest that perfect background matching is not necessary for increased survival. Our results with fish predators are similar to those found with avian predators, where the disruptive elements of moth body patterns (in particular, the spatial positioning of highly contrasting elements across the body) provided increased survival (or reduction in likelihood of attack) without perfect background matching (Schaefer and Stobbe, 2006; Stevens et al., 2006). Additionally, our study suggests a supplementary benefit to having a body pattern that is both background matching and disruptive [so-called differential blending (Cott, 1940)], as the disruptive colouration provides crypsis on a range of backgrounds, possibly due to the varying angle and width of the highly contrasting black and white bars within the body pattern. Humbugs are therefore likely to be protected from detection by fish predators when they are viewed against a range of spatial frequency backgrounds, supporting the idea that disruptive elements are particularly important in concealing animals that reside in heterogenous environments (Thayer, 1909). Indeed, the humbug lives around and within several species of branching corals that are likely to have a range of spatial frequencies (Sale, 1972).

Higher spatial frequency background decreases the likelihood of attack

It was particularly interesting to us that a higher spatial frequency background provided as much (or even greater) reduction in the likelihood of predatory attack than backgrounds with a similar spatial frequency to the humbug body pattern. Our experiment was asymmetrical in design (i.e. the wider-barred backgrounds were comparatively wider than the humbug body pattern in comparison with the narrow-barred backgrounds), and it may be that a slight mismatch in a lower spatial frequency background would provide a similar degree of protection from predation. However, there is evidence from other behavioural experiments that backgrounds with a greater degree of complexity (or greater spatial frequency) could be distracting to the visual system of the predator (Dimitrova and Merilaita, 2009) and, indeed, avian predators take longer to find prey on more complex backgrounds, regardless of the spatial frequency contrast (Dimitrova and Merilaita, 2011). This suggests that a greater number of elements within the background transfers visual attention away from the target or prey.

Additionally, however, a slight mismatch with the background may also enhance the disruptive effect of the humbug body pattern by decreasing predatory edge detection. Edge detection is a key phase in early visual processing in vertebrates, occurring in the RGCs (Land and Nilsson, 2012). Body form can be detected by luminance contrast between the animal and the background (Canny, 1986; Hubel and Wiesel, 1962; Marr and Hildreth, 1980). However, the disruptive vertical bars of the humbug body pattern would create 'false edges' that break-up the body shape of the fish, termed coincident disruptive colouration (Cott, 1940). In frogs, highly contrasting body patterns and enhanced borders unlike those seen in natural scenes have also been shown to act as a camouflage strategy due to the lack of suitable detection methods in predator visual systems (Osorio and Srinivasan, 1991). The disruptive effect may be greater on a background that contains more 'natural edges' than the body pattern (as found on a higher spatial frequency background),

as the contrast between the body outline and background would be further blurred, causing a greater visual illusion to the predators. In which case, a background that was similarly mismatching but lower in spatial frequency may provide a similar degree of protection, and is worth investigating in future studies on animal camouflage.

Viewing distance and disruptive colouration

Our experimental trials, and subsequent Fourier analyses were performed at a set predator viewing distance of 100 cm. With increased viewing distances (similar to those used by coral trout when attacking prey in the wild) it is likely that the black and white bars on the humbug merge into one grey object, as is predicted with many of the bright and intricate reef fish colour patterns (Marshall, 2000; Vorobyev et al., 1999; Wilkins et al., 2016). Indeed, as the reef contains many dappled shadows and grey areas, merged grey objects are likely to be well camouflaged through a direct match to background luminous intensity. We took an underwater field image of humbugs against a coral head and blurred it to examine how well humbugs would match the natural background at a range of viewing distances. To human visual systems, humbugs will remain conspicuous even at a distance of 1000 cm in clear ocean water. However, when modelled using the predators' visual acuity, the effectiveness of the humbug's camouflage increased with increasing viewing distance, until the spatial frequency of the humbug and coral appear to be similar at a distance of 500 cm.

Coral trout ambush prey from a distance of a few metres and therefore it is likely that the combination of their limited spatial acuity, the visual complexity of the reef background and the unique characteristics of the humbug body pattern, allow this species to avoid being eaten as often as other damselfish species, as suggested by gut contents analyses (John, 1995, 1999, 2001). The slingjaw wrasse approaches prey at close distances, suggesting that other aspects of the humbug's ecology play a role in avoiding predatory attacks, such as sheltering within the coral head or the effect of movement combined with the disruptive body pattern. Additionally, the pattern could serve to provide communication between intra-specific individuals while remaining camouflaged at a distance to predators, as a low spatial frequency target pattern is likely to blend into the higher spatial frequency background at distance.

Visual acuity of predators

Our backgrounds were designed to be at the limit of spatial discrimination for both predators, and therefore the predators may have been unable to distinguish the individual bars within the background, due to behavioural visual resolution being lower than theoretical resolution, as demonstrated in other fish (Champ et al., 2014). We calculated θ using the distance between two cone photoreceptors (both double and single cones), and did not take into account potential summation within the RGC layer, optic nerve or optic tectum. Further processing may reduce the spatial acuity of the predator (Collin and Pettigrew, 1989), thereby enhancing the cryptic potential of the prey body pattern against the background. Additionally, lower light levels would encourage regional summation of the signal within the retina, further reducing visual acuity. As the light levels in this experiment were high (all the experiments were carried out during daylight hours), it is unlikely that summation had a significant effect on the visual acuity of the predators.

Movement and orientation of prey

Like many fish, humbugs do not always swim at the same orientation to the coral. We showed that humbugs still retained a

significant reduction in predation risk even when they were not completely aligned with the background habitat. Indeed, the orientation of an animal against a background can enhance or reduce the individual's crypsis, depending on the alignment of features within the background and body pattern (Webster et al., 2009). It has been shown that in some species of moth, individuals rest in non-random orientations that maximise crypsis with the background (Endler, 1984). Moths will change their orientation to a more cryptic position (Kang et al., 2013) using both visual and tactile information (Kang et al., 2015), thereby reducing predator detection (Webster et al., 2009) and suggesting an adaptive advantage of being in-phase with the background. Our results agree to some extent with the alignment hypothesis: the humbug only suffered increased predation when its patterns were perpendicular (90 deg) to the background, which would be rare in the natural environment.

In this study, we did not incorporate movement, and although high-contrast markings are likely to increase predator search times in comparison with low-contrast markings independent of motion (e.g. Dimitrova et al., 2009), high-contrast patterns are hypothesised to increase protection when moving (Allen et al., 2013; Thayer, 1909). This 'motion dazzle' (sensu Cott, 1940)) has been shown to be particularly effective when the pattern is striped or barred as opposed to spotty or plain (Stevens et al., 2008) and can affect the distance travelled, speed and directional information relayed to the viewer's visual system (Conway and Livingstone, 2005; Jackson et al., 1976; Murakami et al., 2006). It has been suggested that, in terrestrial animals, bars and stripes in body patterns may distract predators' visual systems and provide misinformation about the direction of movement of a group of animals (How and Zanker, 2014). However, some studies have shown that the details of the pattern (i.e. whether background matching or disruptive) are not influential in decreasing predation likelihood, as long as the pattern is at least similar to the background. It is the presence of other similarly patterned objects that increase protection (Hall et al., 2013; Stevens et al., 2011). The humbug has a body pattern similar to that of a zebra and congregates in small groups, suggesting an increased confusion effect due to the presence of similarly patterned objects (both humbug and branching coral background). When combined with the attenuating properties of water, it is likely that movement will only serve to increase the camouflage potential of the humbug body pattern. Clearly this is an area that warrants further research.

Limitations of the study
In this study, we have looked exclusively at the humbug and background in greyscale and have not incorporated colour into any visual models. This allowed us to look exclusively at the role of pattern contrast in both background matching and disruptive colouration. As the humbug is black and white, and coral is likely to vary in spectral reflectance between individual colonies, there will be colour contrast between the coral background and humbug body pattern, particularly as many coral reef fish predators are likely to have functional colour vision (Losey et al., 2003; Marshall et al., 2003a,b; Phillips et al., 2016). It would be interesting to identify whether colour adds another element of disruption to the humbug body pattern, and recent methods have been developed to answer exactly these types of question (Endler, 2012). Our experiments do not allow us to directly compare the benefits of background matching versus disruptive camouflage, and we did not design them to do this. Future experiments, however, could incorporate methods to attempt to disentangle these two camouflage strategies, although

there are several methodological obstacles to overcome initially (Webster et al., 2013, 2015).

Conclusions
In conclusion, the results of this study are the first to show quantitatively that although coral head backgrounds serve to increase the crypsis of disruptive colouration, it is not achieved through matching the spatial frequency of the background as previously assumed (Cott, 1940). Instead, a slight mismatch to a higher spatial frequency background enhances the crypsis of disruptive colouration. Therefore, humbugs should be found in environments with a variety of spatial frequency backgrounds, including many higher spatial frequency backgrounds. Further research should identify whether humbugs actively choose higher spatial frequency backgrounds to reduce the likelihood of detection when approached by predators, as has been suggested in killifish (Kjernsmo and Merilaita, 2012).

APPENDIX
Wholemount technique
Five slingjaw wrasse and five coral trout were euthanised individually by placing them in a seawater bath containing 0.20 ml clove oil per litre of seawater (according to ethics approval QBI/192/13/ARC). Retinas from each fish were extracted and fixed in 4% paraformaldehyde immediately using standard methods (Coimbra et al., 2012; Coimbra et al., 2009; Ullmann et al., 2012). For optimal photoreceptor viewing and counting, all retinas required bleaching to remove the densely pigmented retinal pigment epithelium (RPE) that covered large parts of the retina. Bleaching followed similar protocols for other fish and cephalopods (Talbot and Marshall, 2011; Ullmann et al., 2012), in which retinas were submerged for up to 2 h in a 6% hydrogen peroxide solution (in PBS, pH 10) followed by several rinses in fresh, chilled PBS to remove all traces of the oxidative solution (Ullmann et al., 2012). Any remaining vitreous was removed and relaxation slits were cut around the periphery to allow the tissue to lie flat. Retinas were then mounted photoreceptor-side up on slides with a spacer of appropriate thickness (using 1–4 layers of masking tape) to maintain photoreceptor orientation and spacing. Before counting, photoreceptors were cleared in 100% glycerol and the coverslip sealed to the spacer and slide using colourless nail varnish.

Photoreceptors were counted using the Optical Fractionator workflow within the software StereoInvestigator (v11.03; MBF Bioscience) following standard methods to count retinal cells in whole-mounted fish retinas (Coimbra et al., 2012; Coimbra et al., 2009; de Busserolles et al., 2014b). Topographical maps were produced using a script in R developed in previously published work, with the Gaussian Kernel Smoother method that utilises Spatstat (Baddeley and Turner, 2004; Garza-Gisholt et al., 2014). The sigma value was set as the average distance between points (i.e. the average grid size).

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Table S1. The number of trials that each predator completed for each experiment. The predator identity also includes the month and year that the experiments were completed. JF = Jan-Feb; MJ = May-June; 12 = 2012; 13 = 2013; 14 = 2014.

Species	Experiment	Predator identity	Number of trials completed
Epibulus insidiator	1	AJan14	18
		AJul13	18
		BJan14	18
		CJan14	18
		DJan14	18
		EJan14	18
	2	AJan14	18
		AJul13	18
		BJan14	18
		CJan14	18
		DJan14	18
		EJan14	18
	3	AJan14	18
		AJul13	18
		BJan14	18
		CJan14	18
		DJan14	18
		EJan14	18
	4	AJan14	18
		BJan14	18
		CJan14	18
		DJan14	18
		EJan14	18
	5	AJan14	18
		BJan14	18
		CJan14	18
		DJan14	18
		EJan14	18
Plectropomus leopardus	1	DJan14	18
		EJan14	18
		BJF13	18
		BMJ12	17
		DJF13	18
	2	AJF12	30
		AJF13	18

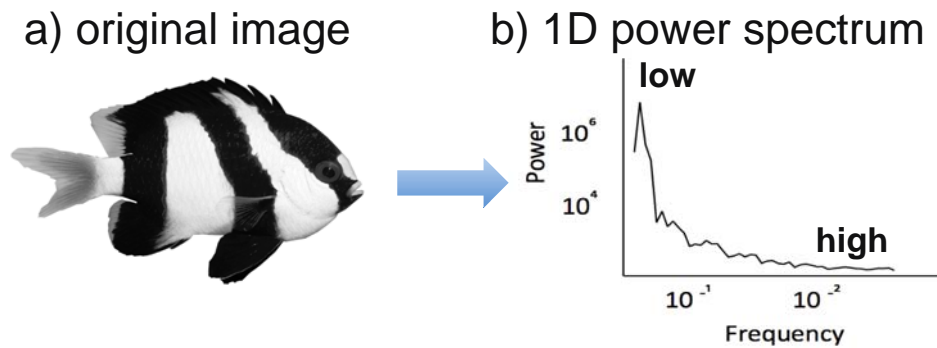|		
	BJF12	**18**
	BJF13	**17**
	CJF12	**18**
	DJF12	**18**
	DJF13	**18**
	FJF13	**30**
	AJF13	**18**
	BJF13	**22**
3	**CJF13**	**19**
	DJF13	**14**
	EJF13	**18**
	FJF13	**18**
	AJan14	**18**
4	**BJan14**	**18**
	CJan14	**18**
	DJan14	**18**
	AJan14	**18**
5	**BJan14**	**18**
	CJan14	**18**
	DJan14	**18**

Fig. S1. Fast Fourier Transformation (FFT) of: A) an image or object within an image. The power spectrum is obtained (B) which shows a 1D 'power spectrum' graph showing the power of different frequencies within an image. The slope of this graph or the peak frequencies within this graph are used to compare the spatial frequencies contained within specific objects or images.