

Visual Acuity in a Species of Coral Reef Fish: *Rhinecanthus aculeatus*

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Key Words

Visual acuity · Sensory ecology · Photoreceptors · Ganglion cells · Cone density · Teleost · Vertebrate eye · Retinal topography · Retinal wholemount · Spatial resolution

Abstract

Coral reef fish present the human observer with an array of bold and contrasting patterns; however, the ability of such fish to perceive these patterns is largely unexamined. To understand this, the visual acuity of these animals – the degree to which they can resolve fine detail – must be ascertained. Behavioural studies are few in number and anatomical analysis has largely focused on estimates of ganglion cell density to predict the visual acuity in coral reef fish. Here, we report visual acuity measures for the triggerfish *Rhinecanthus aculeatus*. Acuity was first assessed using a series of behavioural paradigms and the figures were then contrasted with those obtained anatomically, based on photoreceptor and ganglion cell counts. Behavioural testing indicated an upper behavioural acuity of 1.75 cycles · degree⁻¹, which is approximately the same level of acuity as that of the goldfish (*Carassius auratus*). Anatomical estimates were then calculated from wholemount analysis of the photoreceptor layer and Nissl staining of cells within the ganglion cell layer. Both of these anatomical measures gave estimates that were substantially larger (7.75 and 3.4 cycles · degree⁻¹ for the photo-

receptor cells and ganglion cells, respectively) than the level of acuity indicated by the behavioural tests. This indicates that in this teleost species spatial resolution is poor compared to humans (30–70 cycles · degree⁻¹) and it is also not well indicated by anatomical estimates.

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Introduction

Spatial resolution (acuity) is one of the fundamental measurements used to assess the ability of an animal to discriminate fine detail. The degree to which an organism can perceive detail is important for understanding many aspects of its behaviour and lifestyle. The eagle, for example, enjoys a far greater level of acuity (140 cycles · degree⁻¹) [Reymond, 1987] than the flatworm (0.14 cycles · degree⁻¹) [Land, 1981]. The acuity of the rest of the animal kingdom lies somewhere between these two extremes and depends on the organism's particular lifestyle and environment. A human with 20/20 vision, for example, can resolve objects with a behavioural visual acuity of 30 cycles · degree⁻¹ [though human acuity has been behaviourally found to be much higher in many test subjects; for a review, see Fahle, 2002]. Teleost fish occupy a vast array of niches and lifestyles and necessarily have very varied abilities for spatial resolution [for a review, see

Douglas and Hawryshyn, 1990]. Many deep-water species living in environments that are devoid of sunlight may have very poor acuity – their eyes may instead be more adapted for light detection. Conversely, many shallow-water fish, where light is more freely available and patterns are more complex, may have much greater acuity levels.

Assessment of acuity is undertaken in two ways: behaviourally and anatomically. The most common method of measuring behavioural acuity is to train an animal to distinguish square-wave gratings, made up of alternating black and white bars of equal width. The animal is conditioned to distinguish between vertical and horizontal gratings [Yamanouchi, 1956; Nakamura, 1968; Wilkinson, 1972; Hodos and Yolen, 1976; Yolen and Hodos, 1976] or alternatively a uniform grey stimulus which matches the grating in brightness [Brunner, 1934; Barends et al., 1960; Penzlin and Stubbe, 1977]. The number of bars presented on the stimulus (i.e. the spatial frequency of the pattern) is then increased until the animal can no longer distinguish the stimuli. Optomotor responses and other methods based on the innate responses of the animal have also been used to ascertain the behavioural acuity of an animal [Baburina et al., 1968; Rahmann et al., 1979; Neave, 1984]. These types of tests generally involve placing the animal in a round chamber surrounded by an external cylinder with black and white stripes similar to those used in gratings printed on the inside. This external cylinder is then rotated and the animal is observed to see if its swimming speed and direction vary with the speed of the external drum. If it is unable to see the lines, then its swimming should be unaffected by the moving lines. One of the problems with this type of testing is that it may be affected by the animal's ability to respond to motion as well as its acuity [Muntz, 1974; Westheimer and McKee, 1975; Neave, 1984]. Acuity testing that involves the reaction distance to food items or prey [Hairston et al., 1982; Breck and Gitter, 1983] is similar in that there might be factors other than visual acuity involved in recognizing food. Given the issues with various methods of behavioural acuity testing, multiple paradigms may be appropriate to give a better estimate of acuity in a given species. For this reason, our test species was initially tested with gratings. However, given that the fish had a preference for poking circular stimuli in previous experiments [Pignatelli et al., 2010], a modified version of the paradigm used by Weiler [1966] was also tested; it tested acuity using different-sized dots rather than lines.

Anatomically there are two factors that impose limits on the resolving ability of an animal: the optical proper-

ties of the eye and the spacing of the photoreceptors and retinal neurons. In fish, the optical properties of the eye, i.e. pupil diffraction, aberration of the lens and light scatter in the optical media, are generally very good. Fernald and Wright [1985] found that in African cichlids the resolution allowable by the lens was 10 times greater than that of the retinal grain. Defective focusing of the image on the retina is also unlikely to set a limit to acuity as most fish species are capable of excellent accommodation [Sivak, 1980].

In terms of morphology, the retina of fish is similar to that of other vertebrates, being comprised of 10 layers and containing the same generalized cell types. The specialized cells in the retina receive and analyse light and process these signals for the rest of the visual system. Two of these layers, i.e. the photoreceptor layer and the ganglion cell layer, are particularly associated with acuity. The photoreceptor layer, in the outer retina, is the first processing layer of the retina and contains the cone photoreceptors. The simplest measure of potential acuity in an animal is the spacing between these cone photoreceptors. In theory, if an animal was looking at a black and white grating the light and dark bars of the finest resolvable grating will fall on alternate rows of cones. Thus the minimum resolvable angle would be the angle subtended at the nodal point of the eye by two adjacent cones in the retina. This was first proposed by Helmholtz and König [1896] and in many cases appears to estimate acuity quite well [Northmore and Dvorak, 1979; Neumeyer, 2003; Northmore et al., 2007].

Ganglion cells near the inner surface of the retina are seen to be the final stage of retinal processing. Hence, they are thought to set a limit on the visual acuity of an animal; however, convergence of visual information between the photoreceptors and ganglion cells may also affect the resolution of an image through signal summation [Lythgoe, 1979; Warrant, 1999]. Where many photoreceptors converge onto a single ganglion cell, high signal summation should occur. This may increase the signal integration area, enhancing the retina's ability to detect light at the expense of spatial resolution [Rodieck, 1973]. Where few photoreceptors converge on a ganglion cell, little signal summation should occur and spatial resolution should benefit at the expense of sensitivity. It has been shown that this can be an adaptive process as well, with more ganglion cells being recruited under higher light levels [Northmore, 1977]. Depending on the lifestyle and light environment of a given species, convergence ratios can vary considerably. Many elasmobranchs have ratios ranging from 30:1 to 70:1 (photoreceptors:ganglion cells)

[Litherland et al., 2009], whilst cichlids commonly have convergence ratios of approximately 5:1 [Meer et al., 1984] and goldfish likely have convergence ratios of 2:1 or possibly 1:1 [Neumeyer, 2003], similar to that found in the human fovea [Fahle, 2002].

Anatomy has been extensively used to estimate acuity and there are many fish species in which behavioural acuities are below those of the anatomical estimates. This discrepancy has been found in the skipjack tuna [Tamura and Wisby, 1963; Nakamura, 1968], the convict fish [Yamanouchi, 1956; Nakamura, 1968], the bluegill [Vinyard and O'Brien, 1976; Luecke and O'Brien, 1981a; Williamson and Keast, 1988] and the white Crappie [Browman et al., 1990]. Some of these fish also had anatomical acuities (estimated with both photoreceptor and ganglion cell spacing) several times greater than those behaviourally observed. This may be related to the convergence ratios in the retina of the animal [Litherland et al., 2009] or possibly a limit imposed later in the visual system. The opposite is true for humans, where behavioural acuity is better than retinal anatomy would suggest. The ability of humans to seemingly exceed anatomical limits has been termed 'hyperacuity'. Thus, to obtain a more accurate idea of the acuity of an animal, both the behaviour and the anatomy of the animal should be investigated.

Here we present an integrative approach to the study of acuity in a species of reef fish. We investigated behavioural acuity as well as the inter-cone spacing, ganglion cell spacing and convergence between the photoreceptor and ganglion cell layers. *Rhinocanthus aculeatus* was chosen as the test species not only because of its highly patterned exterior but also because of recent studies that suggest trichromacy in this species [Pignatelli et al., 2010] and detail the bipolar cells in its inner retina [Pignatelli and Marshall, 2010] which are indicative of a potentially good visual ability. *R. aculeatus* is a common triggerfish living on subtidal reef flats and in shallow lagoons along the Indo-Pacific coasts where light is abundant. This species also displays a number of large and small stripe patterns across its entire body, perhaps for purposes of communication on the reef, which could be indicative of a reasonable degree of visual acuity.

Methods

Animals

All animals were treated in accordance with the ethical guidelines of The University of Queensland (AEC approval No. SBS/738/08/ARC). Ten juvenile specimens 7–12 cm in size were collected around Lizard Island, Northern Queensland, Australia.

In preliminary tests, adult fish had proved hard to train due to aggressive behaviour – often destroying the test apparatus. Fish were kept in individual aquaria and fed with fish flakes (Flake Frenzy; HBH Enterprises, Springville, Utah, USA) prior to testing. The water temperature in the aquaria was kept constant at 23–26°C. The aquaria were lit with standard 60-Watt fluorescent tubes and 60-Watt Samsung black lights, to simulate the spectrum of natural sunlight.

Experimental Set-Up, Training and Behavioural Tests

Behavioural visual acuity was tested using a two-choice training experiment with a food reward. The fish had to discriminate two identical square-wave grating stimuli (fig. 1). These were manufactured as black and white lines printed on photo paper (EPSON matte paper – heavyweight, 167 g/m²), cut to size (4 × 4 cm) and then laminated (80-µm laminate), so they could be presented in water. One stimulus was orientated with the gratings vertical and the other with the gratings horizontal. The stimulus size was kept constant (4 × 4 cm) for all tests and only the number of cycles on the stimuli changed.

Initially, fish were tested to see if they could be trained to reliably discriminate a horizontal square-wave grating from a vertical one. Two stimuli (each with 23.5 cycles printed on them) were lowered into the tank. Half the fish were trained to 'tap' the vertical stimulus and half were trained to tap the horizontal one. This was to see if there was any innate preference for vertical or horizontal stimuli in this species. Fish were rewarded with food from a syringe if they tapped the stimulus they were trained to tap. The fish were presented with the reward or left unrewarded on the left or right side of the test apparatus in a random fashion. The fish were trained until they could discriminate the stimuli at a level of at least 80% correct. It took approximately 4 weeks to train all of the test fish to discriminate at this level.

For the acuity tests, a modified Y-maze was built (fig. 1) to control the distance at which the fish made their decision. This was necessary to ensure that fish observed the stimuli with a given visual angle. The main components of the Y-maze consisted of a central divider, a test board on which the stimuli were affixed and a clear transparent barrier (acrylic Perspex, 2 mm thick). The central divider created two compartments (left and right) which the fish observed from behind the barrier. It also positioned the fish at a known distance away from the test stimuli. At the start of every trial the test board was slid into position, placing one of the stimuli in each compartment. The fish were allowed to observe the two compartments from behind the barrier for 10 s, at which point the barrier was raised. The fish then had to make a choice for one of the stimuli by swimming to it and 'tapping' on the stimulus. If the fish successfully identified the reward stimulus, a syringe of food was lowered into the back of the tank and the fish were given a small portion of food. Furthermore, the Y-maze was constructed so that the centre divider could be removed and replaced with another centre divider of a longer length. This meant that the distance at which the fish were forced to make a choice could be changed.

Experiment 1: Square-Wave Gratings

Individual fish were tested with square-wave gratings at 3 distances, i.e. 10, 20 and 30 cm, which were positioned such that they were centred on the backboard of the Y-maze. Seven fish were tested at the 10-cm distance, 6 at 20 cm and 5 at 30 cm. Each indi-

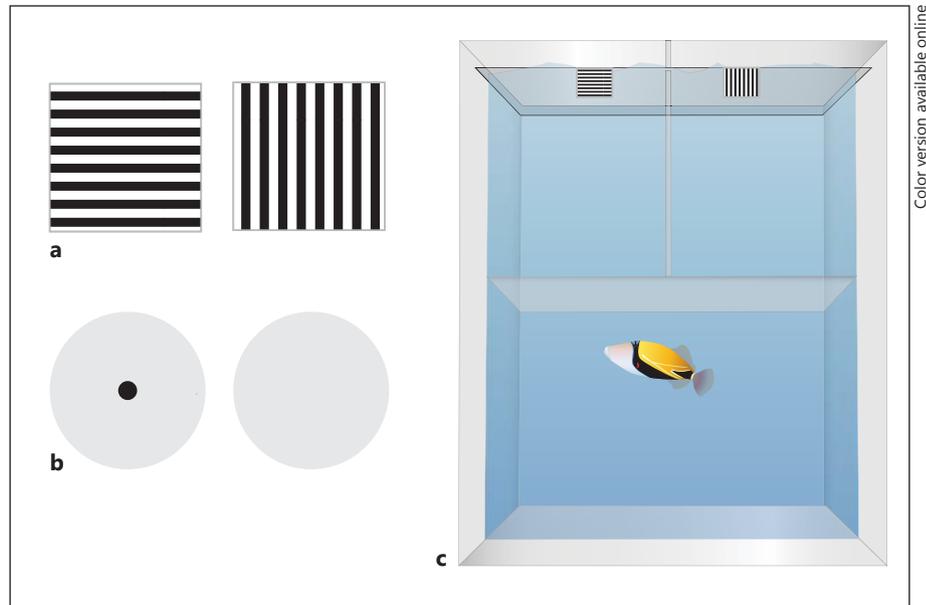


Fig. 1. Stimuli sets used in the experiment and Y-maze set-up. **a** Square-wave gratings used for the behavioural tests of *R. aculeatus*. One ‘cycle’ consists of a black and white lined pair – hence, these stimuli each display 7.5 cycles. Animals were trained to discriminate a stimulus of one orientation, vertical or horizontal, from the other. **b** Circle stimuli used in the second set of behavioural experiments. Two circle stimuli were presented to the fish: a rewarded stimulus with a smaller dark circle printed in its centre and an unrewarded stimulus that did not have a dark smaller circle

in its centre. The fish were trained to poke at the stimulus with the dark smaller circle. **c** Y-maze behavioural set-up. The fish were kept at a given distance from the stimuli via the central divider and the transparent barrier. In this way, the fish were forced to make a choice for one stimulus over the other at a given distance. Fish were given 10 s to view the stimulus in each compartment before the transparent barrier was lifted and the fish was allowed to make a choice.

vidual fish was given just 20 trials per day to keep the fish interested in the task. In total, each individual fish was given 100 trials at every acuity level and distance. When the fish had completed the 100 choices at a given acuity level, stimuli with a higher spatial frequency were introduced until the fish could no longer distinguish the vertical stimulus from the horizontal one. The threshold criterion was set at 65% correct – below this, a fish was taken to be unable to reliably discriminate the stimuli.

Experiment 2: Circle Stimuli

After testing with the square-wave gratings, the fish were trained using circle stimuli. Two circular stimuli were presented to the fish in the Y-maze following the same procedure as with the square gratings. Both stimuli were identical achromatic circles (RGB values: 225, 225 and 225) 4 cm in diameter; however, one of the stimuli had another smaller circle (RGB values: 25, 25 and 25) printed in its centre (fig. 1). Seven fish were trained to identify and tap this smaller circle to receive a food reward. Initially, 3 sizes of ‘smaller circles’ were tested, measuring 3, 1 and 0.5 mm in diameter. The fish were presented with each size stimulus in the Y-maze 50 times at a distance of 10 cm. Additionally, 3 fish were subsequently tested 100 times with circle stimuli measuring 1.5 and 0.7 mm in the Y-maze, once again at a distance of 10 cm. The size of the circle stimuli was then converted into cycles · degree⁻¹ by assuming that the diameter of the smaller circle represented one half of a cycle.

Statistical Analysis of Behavioural Results

Fisher’s exact test was used to compare the results of the fish. The p values given are the values for an individual fish compared against the group average and they are two-tailed. All error bars shown on the graphs are binomial 95% CI.

Anatomical Estimates of Acuity

Following behavioural testing, both the photoreceptor layer and the ganglion cell layers of the retina were examined. The animals were euthanized with an overdose of clove oil according to the guidelines of the University of Queensland (AEC approval No. SBS/738/08/ARC). The dorsal and ventral limbi were scored for orientation and the eyes excised. The cornea and lens were then removed and the retinas dissected free of the eyecup in 0.1 M phosphate buffer solution (pH 7.4). The retinas were then immersion fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h. After fixation, the retinas were wholemounted for analysis following the methods of Stone [1981], Collin and Pettigrew [1989] and Litherland and Collin [2008].

Examination of the Photoreceptor Layer

The retinas were initially mounted with the photoreceptor layer up to assess inter-cone spacing. A thin layer of filter paper was cut to size and used as a border around the retina to prevent the coverslip from flattening the photoreceptors when applied. The retinas were mounted in 100% glycerol and then the coverslip was

applied and sealed with nail polish to prevent dehydration. Preliminary surveys indicated that the rods in *R. aculeatus* were difficult to observe, even using retinal samples that had been dark-adapted for 24 h and were only evident in the periphery of the retina. It was assumed that, given the high-light environment that the fish lived in, the cones, being the photopic photoreceptors, could be taken as being primarily responsible for behavioural acuity tasks. Hence, counting was performed only on cones.

Photoreceptor measurements were performed using a Zeiss AxioPlan II compound microscope fitted with an X-Y-Z motorized stage (BioPrecision, Ludl Electronic Products Inc., Hawthorne, N.Y., USA) and NID optics. A randomly assigned sampling grid was created using Stereo Investigator 6 software (MicroBrightField Inc., Williston, Vt., USA), which controlled stage movement. Counts were made at 0.45-mm intervals which allowed 250–300 sites per retina to be photographed. Pictures were taken at these sites at a magnification of $\times 630$ using NID and then analysed using ImageJ software (rsb.info.nih.gov). Afterwards, each site was visually assessed with a light microscope to ensure that the photoreceptors had not been deformed during the wholemounting process and that they were free of pigment, which would obscure measurement of the cones. During the assessment, only cone photoreceptors were counted. Final counts were multiplied by 0.83 to account for shrinkage during fixation; this ratio has been validated by comparing the shrinkage of fixed and fresh retinas of *R. aculeatus* [Marshall and Champ, unpubl. data]. Topographical maps of the inter-cone spacing across the retina were constructed following the method of Collin and Pettigrew [1988b] and Litherland and Collin [2008]; isodensity contours were drawn by linking areas of similar cell density via interpolation between 4 and 5 points.

Ganglion Cell Layer

Once the photoreceptor isodensity maps of a given retina were completed, the retina was removed from its slide, flipped and re-mounted with the ganglion cell layer facing upward. Retinas were air dried for 24 h, rehydrated in a graded ethanol series and then stained for 7–10 min in 0.1% cresyl violet (pH 3.9). When the retinas had reached the correct level of staining, observable under a light microscope as a dark purple stain, they were washed with de-ionized water then dehydrated through the a graded series of ethanols. Finally, they were cleared in xylene and a coverslip was applied with Depex mounting medium. The ganglion cell distribution and densities were once again surveyed with the Zeiss AxioPlan II compound microscope following the same method described for the photoreceptor layer.

To identify the numbers of ganglion cells present in the retina, the ganglion cell layer was examined for cells stained dark purple by the cresyl violet stain. Ganglion and amacrine cells were distinguished from glial cells by the presence of cytoplasmic staining. These cells were also distinguished by their large irregularly shaped profiles. This classification has been validated for teleosts [Collin and Pettigrew, 1988a, b], where cells have been identified by retrograde labelling from the optic nerve and compared with Nissl-stained cell profiles. Following the method of Collin and Pettigrew [1988a], final counts were multiplied by 0.76 to account for any displaced amacrine cells in the ganglion cell layer that were also stained by cresyl violet. This ratio has been shown to be an effective means of estimating the ganglion cells in teleosts via comparisons of Nissl-stained ganglion cells and those stained via retrograde labelling from the optic nerve [Collin and Pettigrew, 1988a].

Anatomical Acuity: Inter-Cone Spacing, Ganglion Cell Spacing and Retinal Summation

Following the methods of Collin and Pettigrew [1989] and Litherland and Collin [2008], inter-cone spacing, ganglion cell spacing and summation ratios were calculated using the observed photoreceptor and ganglion cell counts. *R. aculeatus* was found to have a square photoreceptor mosaic across its retina, with double cones outnumbering single cones 2:1. Calculations of acuity based on inter-cone spacing therefore followed the method of square mosaics where the spatial resolution is calculated by obtaining the number of cells subtended by 1° of visual arc. Methods for this followed those of Collin and Pettigrew [1989] where using the ratio of Matthiessen we can calculate the distance from the lens centre to the retina (posterior nodal distance; PND). *R. aculeatus* was found to have a lens diameter of $2.67 \pm .28$ mm ($n = 20$), hence:

$$\text{PND} = 2.55 \times 1.335 \text{ (radius)}$$

$$\text{PND} = 3.4 \text{ mm}$$

Then, the angle subtending 1 mm on the retina can be calculated as follows:

$$\tan \alpha = \frac{1 \text{ mm}}{\text{PND}}$$

$$\alpha = \arctan \frac{1}{3.40425}$$

where $\alpha = 16.37^\circ$.

Resolving power for the inter-photoreceptor estimate assumed that all of the photoreceptors in the mosaic were being used for the animal's visual acuity.

Retinal Summation

Summation ratios are inversely related to photoreceptor and ganglion cell spacing counts, with the lowest summation ratios observed in regions of peak cell density. Once the isodensity maps of the photoreceptor and ganglion cell layers were constructed, these two maps were overlaid and convergence was calculated as the number of photoreceptor cells/number of ganglion cells within an area of 1 mm^2 , following the methods of Litherland and Collin [2008] and Temple et al. [2013]. There were no noticeable signs of shrinkage on the retina after it had been through the graded ethanol series, cleared and stained with cresyl violet.

Results

Behavioural Testing: Square-Wave Gratings

Seven fish named fish 1 to fish 7 were presented with the horizontal and vertical stimuli in the Y-maze at a distance of 10 cm, i.e. 4 with the vertical stimulus as the reward and 3 with the horizontal stimulus as the reward. Acuity was tested starting at $1 \text{ cycle} \cdot \text{degree}^{-1}$ and increased in increments of approximately $0.25 \text{ cycles} \cdot \text{degree}^{-1}$ up to $1.75 \text{ cycles} \cdot \text{degree}^{-1}$, at which point the discrimination rate for the group fell below 65%. This threshold criterion was picked as the cut-off for reliable discrimination following consideration of previous be-

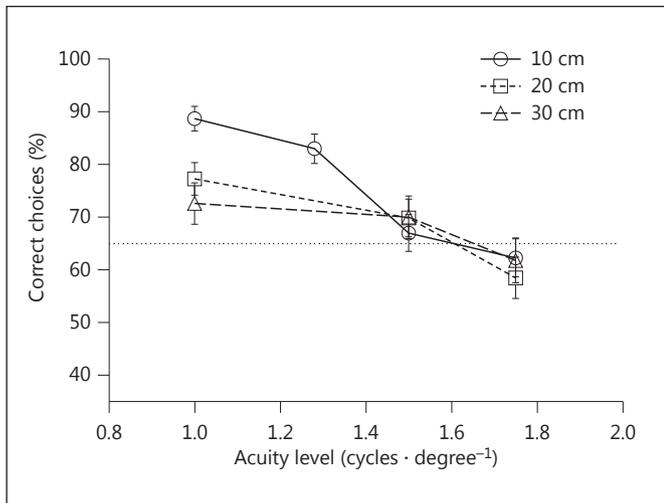


Fig. 2. Group result of the square-wave gratings tests. Fish were able to discriminate gratings to a level of approximately 1.75 cycles · degree⁻¹, at which point they dropped below the threshold criterion of 65%.

havioural studies of animal vision [Neumeyer, 2003; Kohitaka et al., 2008]. All 7 fish successfully discriminated the 1 cycle · degree⁻¹ gratings with an average choice frequency of 88.7%. The choice frequency then rapidly dropped off until the fish fell to 62.3% choice frequency when tested with the 1.75 cycles · degree⁻¹ gratings (fig. 2). Only fish 7 had results that were significantly different for the 1 and 1.25 cycles · degree⁻¹ gratings ($p = 0.0339$ and $p = 0.0001$, respectively), scoring 81% with the 1 cycle · degree⁻¹ grating and 62% with the 1.25 cycles · degree⁻¹ grating at this distance. Fish 2 was the only fish with significantly different results for the 1.5 cycles · degree⁻¹ ($p = 0.0025$) and 1.75 cycles · degree⁻¹ gratings ($p = 0.0011$), scoring 94% correct choices in the 1.5 cycles · degree⁻¹ testing and 82% correct choices in the 1.75 cycles · degree⁻¹ testing. The scores were significantly better than the average for these tests, i.e. 83 and 67%, respectively.

Six fish (fig. 2) were tested with the 1, 1.5 and 1.75 cycles · degree⁻¹ gratings at a distance of 20 cm. The fish as a group could discriminate the 1 and 1.5 cycles · degree⁻¹ stimuli; however, they once again fell below the threshold criterion of 65% when tested with stimuli at a level of 1.75 cycles · degree⁻¹. There was no statistically significant difference in the individual results of the fish for any of the stimuli tested at 20 cm ($p > 0.05$). Five fish were then tested once again with the 1, 1.5 and 1.75 cycles · degree⁻¹ gratings at a 30-cm distance. The fish again fell below the

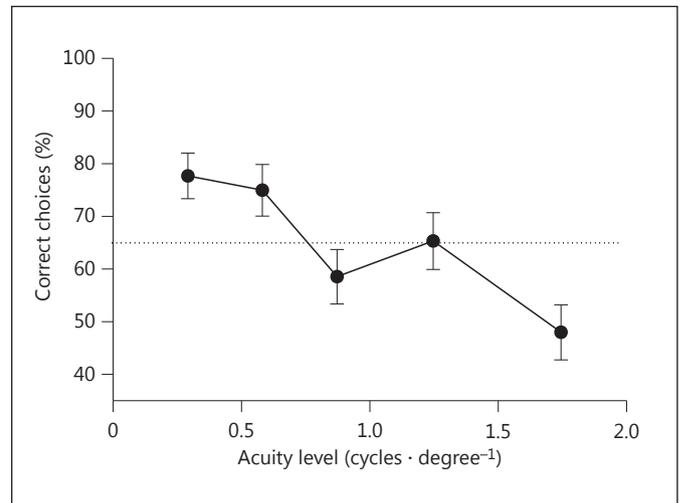


Fig. 3. Individual results of the circle stimuli tests and the group result. A group fish appeared to drop below the threshold criterion of 65% correct choices close to 1 cycle · degree⁻¹. If the threshold criterion is dropped to 50%, the fish appear to stop being able to discriminate at a level of 1.6–1.75 cycles · degree⁻¹.

65% threshold criterion at 1.75 cycles · degree⁻¹. There was no difference in the individual results of the fish for the 1-cycle gratings tests at this distance; however, for the 1.5 cycles · degree⁻¹ test, fish 4 scored 59% correct choices, which was significantly different ($p = 0.0364$) from the scores of the other fish which averaged 72.5%. In the 1.75 cycles · degree⁻¹ test, fish 1 was the only fish which achieved individual results that were significantly different ($p = 0.0138$) from those of the other fish, scoring 48% correct choices for this test whilst the other fish recorded an average of 65.25%.

Finally, two random fish were chosen to be tested with a 0.8 cycles · degree⁻¹ stimulus to ascertain if motivation for the task remained. Fish were given 50 trials each, in which they successfully identified the reward stimuli with choice frequencies of 88 and 96%, respectively.

Behavioural Testing: Circle Stimuli

Seven fish were tested with the circle stimuli at 10 cm in the Y-maze. This was the distance at which the fish had the highest number of correct choices in the square-wave gratings tests. Fish successfully discriminated the stimuli when the dot was 3 mm (0.29 cycles · degree⁻¹) in diameter, with a collective choice frequency of 77.7% (fig. 3). Discrimination for the 1-mm (0.87 cycles · degree⁻¹) and 0.5-mm (1.75 cycles · degree⁻¹) circles fell below the threshold criterion of 65%, with choice frequencies of 58.6 and 48%, respectively.

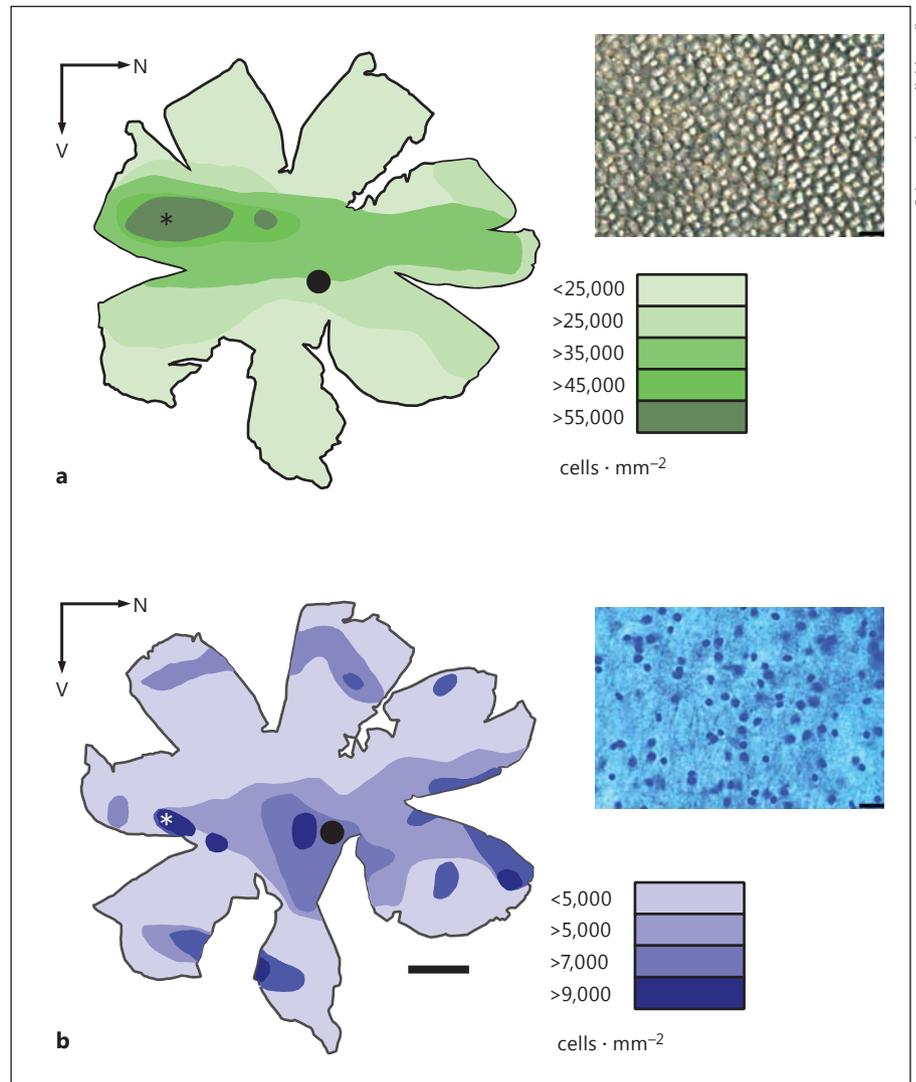


Fig. 4. Isodensity maps of the right eye of one *R. aculeatus*. Photoreceptor (a) and ganglion cell layers (b). The cell density, in general, was greater in the centre of the retina. Photoreceptor cells had a maximum cell density of approximately 64,400 cells · mm⁻² and ganglion cells had a maximum density of 12,450 cells · mm⁻². Both of these high-density regions overlapped in a small area of the temporal region of the retina. N indicates the nasal direction and V the ventral. Scale bars = 10 μm (insets) and 1 mm (a, b). The asterisk indicates the region where the highest cell count was determined.

Three fish were then further tested with 1.5-mm stimuli (0.58 cycles · degree⁻¹) and 0.7-mm stimuli (1.25 cycles · degree⁻¹). Each fish was given 100 choices with both sizes of stimuli. All 3 fish successfully discriminated the 1.5-mm stimulus from the blank stimulus in 75% of the trials. In trials with the 0.7-mm test stimulus, the individual results for 1 fish varied considerably (77%) from the results of the other 2 fish tested (63 and 56%) with this grating and resulted in a group choice frequency of 65%. This result was the only individual choice frequency in any of the circle tests that was significantly different from the results of the other fish ($p = 0.0348$). This individual fish did not perform significantly better in any other circle of the square-wave gratings test. However, if we were to exclude this individual result, the average choice fre-

quency for the reward in the 0.7-mm circle test would have been 59.5% rather than 65%.

Anatomical Resolving Power

Eight retinas from 3 fish were examined to ascertain the areas with the highest photoreceptor cell density present in the triggerfish retina. The retinal pigment epithelium proved difficult to remove even after 24 h of dark adaptation. Only in 2 retinas was the pigment removed satisfactorily enough to construct a complete topographic map of the photoreceptor map across the retina; however, topographic patterns were consistent in both individuals. Acuity estimates based on inter-cone spacing were then calculated using these areas of highest cell density. In the highest acuity region in the *R. aculea-*

tus retina, cones were found to have a density of 64,408 cells · mm⁻². Assuming all cones were used for high-frequency pattern analysis, the photoreceptor-derived estimate was 7.75 cycles · degree⁻¹. Four retinas were then examined to ascertain the highest areas of ganglion cell density. This was consistent for the 4 retinas examined. Ganglion cells were found to have a peak cell density of 12,450 cells · mm⁻² and the acuity estimate derived from this density was 3.41 cycles · degree⁻¹. Only one retina was able to be examined on both the photoreceptor and ganglion sides (fig. 4) and therefore the summation ratio was only able to be effectively calculated for one retina. This was found to be approximately 4:1 in an area where the highest ganglion cell distribution and the highest photoreceptor counts overlapped. The isodensity maps of the cones (fig. 4) are indicative of horizontal streaks in the retina, where the highest areas of ganglion cell density correspond with the areas of high photoreceptor cell densities in the central and temporal regions of the retina. However, the ganglion cell layer forms an incomplete horizontal streak that extends from the nasal to the central retina. Additionally it was found that there were high-density spots outside the incomplete horizontal streak in this layer. The horizontal streaks for the cone layer also appeared to be situated slightly dorsally to the streak in the ganglion cell layer.

Discussion

Behavioural Results

The behavioural acuity found for *R. aculeatus* is similar to that found for freshwater species such as the goldfish [Hester, 1968; Wilkinson, 1972; Neumeyer, 2003] and the bluegill (*Lepomis macrochirus*) [Breck and Gitter, 1983]. Both of these species have had acuities observed to be approximately 1.5–2 cycles · degree⁻¹, measured based on both the heart rate [Hester, 1968] and gratings in the case of the goldfish [Wilkinson, 1972; Neumeyer, 2003] and based on the reaction distance to prey in the case of *L. macrochirus*. It should be noted that the estimates obtained from the experiments of Neumeyer [2003] used threshold criteria (75 and 70% correct choices) that were different from the criterion of this study (65% correct choices). However, the difference in acuity estimates is not great even if we apply our criterion of 65% to the tests of Neumeyer [2003] (approximately 0.25 cycles · degree⁻¹). In tests involving the gratings, once the fish was seen to fall below the 65% threshold the tests were stopped shortly thereafter. The choice to stop

the tests once the fish had fallen below this pre-determined threshold was largely determined by previous studies of acuity in fish and the motivation of the test specimens. In previous studies of the acuity of fish [Nakamura, 1968; Wilkinson, 1972; Neumeyer, 2003] and other animals [Giurfa et al., 1996; Prusky et al., 2000], results below the arbitrary threshold were not taken to be discriminable. For example, in the case of Neumeyer [2003], the limit of acuity is seen to be the point where the fish falls below 70% correct choices. Below this, the number of choices for the reward may still be statistically significant; however, the stimuli are not seen as reasonably discriminable by the test animal – hence, the acuity limit is taken at the pre-determined threshold. Given that below our pre-determined choice frequency threshold (65%) the fish was taken as not being able to distinguish the two grating stimuli to a reasonable degree – testing was stopped once it was below the threshold criterion to prevent demotivation of the fish for further tests in the series. It can be seen that ideally a 50% choice criterion should be reached to determine the point at which the stimuli are completely indistinguishable to the fish. However, given the difficulty obtaining, maintaining and training the fish to do the required task, this was seen as a necessary trade off.

In the circle stimuli tests the fish reached a 50% choice frequency for the reward. The fish appeared to perform more poorly in these tests than in the gratings tests. Fish were seen to fall below the 65% threshold criterion between 0.75 and 1.25 cycles · degree⁻¹, which was worse than the 1.75 cycles · degree⁻¹ limit found with the gratings tests. Interestingly, fish choice for the reward was completely random (50% for the reward) in the tests with the 1.75 cycles · degree⁻¹ circle stimuli. The reason for the discrepancy in the gratings and the circle stimuli tests is unknown; however, the circle stimuli were different from the gratings stimuli in that the perception of the rewarded circle stimulus is essentially a detection task. The rewarded and unrewarded stimuli were not matched for brightness (unlike the gratings stimuli); however, this difference resulted in worse results for the circle stimuli and did not aid detection. It may be that the contrast sensitivity, i.e. the minimum difference in radiance between an object and its perceivable background, is particularly poor in this species. A number of varying results for contrast sensitivity have been found in teleost species [Douglas and Hawryshyn, 1990], complicated by differences in the testing methods used. For the circle tests, though, more than the increased contrast sensitivity of the gratings tests would be beneficial for discriminating the small dot on the reward stimulus.

It has also been seen that different acuity paradigms can result in different results. Bees, for example, have been seen to have different acuities depending on whether they are looking at square-wave or circular gratings [Srinivasan and Lehrer, 1988]. In humans, too, under certain circumstances, there are differences in the acuity results obtained with gratings and with stimuli such as tumbling E letters [Anderson and Thibos, 1999]. Critically, the circular stimuli may also subtend a smaller angle on the retina of the fish than do the gratings stimuli. Hering [1861] famously described ‘hyperacuity’ in humans to be the result of interpolation over many dozens of photoreceptors. This was subsequently disproved in humans by Ludvig [1953] nearly a century later; however, for a fish like *R. aculeatus*, with a convergence ratio larger than that in humans (4:1 rather than 1:1), interpolation over dozens of photoreceptors could possibly improve acuity. Additionally, the larger gratings stimuli may be easier for *R. aculeatus* to ‘focus’ on or ‘image’ on the higher acuity areas of its retina. It is interesting to note that the best results obtained with *R. aculeatus* for gratings were at the closest distance – where the stimuli subtended the greatest angle on the animal’s retina. However, more testing should have to be done to ascertain the effect of stimuli size on acuity in this species.

Anatomical Estimates of Acuity

Examination of the photoreceptor and ganglion cell layers gave estimates of acuity which were higher than those obtained through the behavioural tests. The anatomical estimates indicated an acuity level between 3.4 and 7.75 cycles · degree⁻¹, which indicated a far higher spatial resolution than the behavioural result. A similar gap has been observed in fish species such as the skipjack tuna [Tamura and Wisby, 1963; Nakamura, 1968], the convict fish *Microcanthus strigatus* [Yamanouchi, 1956; Nakamura, 1968], the bluegill [Vinyard and O’Brien, 1976; Luecke and O’Brien, 1981b; Williamson and Keast, 1988] and the white crappie (*Pomoxis annularis*) [Browman et al., 1990]. Conversely, a number of other studies have found a good level of agreement between the behavioural and anatomical estimates in fish [Brunner, 1934; Baerends et al., 1960; Weiler, 1966; Northmore and Dvorak, 1979; Neumeyer, 2003; Temple et al., 2013]. Conclusions made on the basis of these studies are complicated by the fact that few of them measure behavioural acuity as well as photoreceptor and ganglion cell numbers. For the few species that have had all three examined in detail, it has been seen that behavioural acuity is better predicted in species with low convergence ratios between

the photoreceptor and ganglion cells. For example, the behavioural acuity of the bluegill, with photoreceptor:ganglion cell ratios of 13:1 [Williamson and Keast, 1988], is not well predicted whilst estimates for the goldfish (1:1 or 1:2) [Neumeyer, 2003] and the archerfish (1:1) [Temple et al., 2013] are. Presumably, low convergence ratios mean very little spatial information is lost between the photoreceptor and ganglion cell layers [Kock, 1982] and therefore retinal grain is a good indicator of behavioural acuity; however, identification of the ganglion cells responsible for acuity is problematic. In general, there are small and large categories of ganglion cells, with the former being considered responsible for spatial processing [Kock and Reuter, 1978]. The population of large ganglion cells has been found to be small (0.5–5%) in both teleosts and elasmobranchs [Kock and Reuter, 1978; Bozzano and Collin, 2000], and they are generally not excluded in acuity studies but doing so may give a refined estimate of acuity. However, another issue is that small ganglion cells can be further classified into many different types on the basis of dendritic morphology and these differences probably reflect differences in function. In fish, two of these different types, i.e. direction-specific and orientation-specific ganglion cells, have been seen to be important for the detection and resolution of moving and stationary gratings [Maximova, 1999; Maximov et al., 2013]. Finding specific markers for specific types of ganglion cells may open a new avenue for the anatomical estimation of visual acuity. However, in at least one study of the detection of periodic gratings by fish, even when specific ganglion types (direction specific and orientation specific) were considered in isolation they were not seen to act as linear integrators of signals [Maximov et al., 2013]. Hence, the spatial resolution for periodic gratings was not determined by the size or density of their receptive field alone.

The issues with predicting acuity based purely on retinal grain and ganglion cell counts in fish is mirrored in humans, where behavioural acuity is found to exceed the anatomical limits. ‘Hyperacuity’ in humans (who have convergence ratios of 1:1 in the fovea) is well documented and the normal human observer can achieve acuity thresholds far above those suggested by the spacing of adjacent photoreceptors in the fovea and the ganglion cell counts [Westheimer, 1976; Fahle, 2002]. These high levels of spatial acuity are likely achieved because, unlike in teleost fish, the optics of the human eye are relatively poor. Even a point of light from outside the eye projected with a high resolution onto a single photoreceptor produces an airy disk on the retina that has a diameter great-

er than 1 photoreceptor [Campbell and Gubisch, 1966]. If a light stimulus is shifted less than a photoreceptor width, then this is perceivable because the level of activation of the surrounding photoreceptors will change depending on their proximity to the centre of the light stimulus [Fahle, 2002].

Given these considerations, it can be seen that the prediction of behavioural acuity using anatomical measures is problematic. Identification of the key cells in the retina responsible for acuity may better predict acuity. The way in which this spatial information is processed from the photoreceptors to the ganglion cells is probably also important, as is the case in humans. Given that Penzlin and Stubbe [1977] found that binocular acuity in the goldfish is better than monocular acuity, there may also be some central contribution to acuity processing.

Retinal Topography and Environment

The analysis of retinal topography in both cell layers shows a distinct horizontal streak in the photoreceptor layer and a partial horizontal streak in the ganglion cell layer, with the highest acuity areas occurring in the temporal region. The 'terrain theory' [Hughes, 1977] suggests that the topography of cells across the retina is indicative of the symmetry of the perceived world. In species whose visual environment is unobscured, an increase in cells across the middle of the retina should also be observed. This horizontal retinal streak should allow an animal to scan the horizon without the eye movements necessary to focus the image of the world onto the area centralis [Collin and Pettigrew, 1988c]. In theory, it should also allow an animal to perceive movement at a lower threshold. This may be useful to *R. aculeatus* as it spends much of its time in relatively open areas sorting for food in rocky substrate adjacent to the reef [Champ, pers. observation].

The difference in horizontal streak positioning observed in the two retinal layers may in part be due to the age of the animals used for testing. In teleosts the retina continues to grow throughout life [Johns and Easter, 1977; Meyer, 1978], expanding like a balloon with new neurons being added at the retinal margins [Muller, 1952; Johns, 1977; Meyer, 1978; Sharma and Ungar, 1980]. We used juvenile fish as we found them more suitable for behavioural testing; however, it would be interesting to observe the ganglion and photoreceptor streaks in older fish to see if any changes in the streaks are observed with age.

Visual Acuity on the Reef

It is perhaps unlikely that a species that is as highly patterned as *R. aculeatus* should perform so poorly in behavioural tests of acuity. However, it is possible that many of the colourful and contrasting patterns we see on coral reef fish may actually be useful for camouflage. As early as 1917, Longley [1917] observed that many labriform fish are colourful at close distance; however, at range and with intervening water, that colouration is lost due to the fine patterns of the skin becoming blurred. Marshall [2000] examined this colour mixing by taking into account the spectra of the colours displayed in a number of species as well as their spectral sensitivities. He also used anatomical estimates of acuity in reef fish from Collin and Pettigrew [1989], ranging from 7 to 27 cycles · degree⁻¹, to suggest that most colour patterns probably become blurred between 1 and 5 m. This blurring of colours and patterns may actually facilitate inconspicuousness on the reef. The behavioural acuity results of *R. aculeatus* appear to corroborate such assumptions about reef fish patterns. Given its poor acuity, the colour of the animal should blur at relatively close distances and not be effective for long-range communication. Additive mixing with the background spectra coupled with the poor acuity of *R. aculeatus* may mean that at a distance the fish effectively blurs into the background of the coral reef environment, which is spectrally diverse and highly varied like the patterns on the fish.

The results of this study indicate that for at least one species of reef fish there is a discrepancy between acuity estimates derived from behavioural and anatomical measurements. This is similar to the difference in anatomical and behavioural results found in a number of other fish species. Whether this discrepancy is due to mechanisms imposed at the retinal stage such as convergence ratios or 'higher' mechanisms post-retina is unclear. However, it can be seen that in *R. aculeatus*, and perhaps many other reef fish, the finite limit set on behavioural acuity is not set by the ganglion cells or the photoreceptors. As a result, previous estimates of reef teleost acuity based on anatomy can be seen to be estimates of potential acuity but not necessarily definitive thresholds of acuity. Collin and Pettigrew [1989] suggested that acuity is higher in ambush predators on the reef based solely on the ganglion cell densities of such species. It would be interesting to re-examine such species behaviourally to see if these species are also proved to have the highest acuities behaviourally. A true correlation between lifestyle and acuity could then be made. Additionally, it can be seen that the presence of intricate patterns does not necessarily indicate a high lev-

el of acuity in reef species. This is important given that the elaborate patterns displayed by reef fish have long been a focus of interest for many researchers. Such patterns may be a compromise between short-range communication and long-range camouflage as has previously been suggested [Marshall and Vorobyev, 2003].

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