A spitting image: specializations in archerfish eyes for vision at the interface between air and water

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Archerfish are famous for spitting jets of water to capture terrestrial insects, a task that not only requires oral dexterity, but also the ability to detect small camouflaged prey against a visually complex background of overhanging foliage. Because detection of olfactory, auditory and tactile cues is diminished at air–water interfaces, archerfish must depend almost entirely on visual cues to mediate their sensory interactions with the aerial world. During spitting, their eyes remain below the water's surface and must adapt to the optical demands of both aquatic and aerial fields of view. These challenges suggest that archerfish eyes may be specially adapted to life at the interface between air and water. Using microspectrophotometry to characterize the spectral absorbance of photoreceptors, we find that archerfish have differentially tuned their rods and cones across their retina, correlated with spectral differences in aquatic and aerial fields of view. Spatial resolving power also differs for aquatic and aerial fields of view with maximum visual resolution (6.9 cycles per degree) aligned with their preferred spitting angle. These measurements provide insight into the functional significance of intraretinal variability in archerfish and infer intraretinal variability may be expected among surface fishes or vertebrates where different fields of view vary markedly.

Keywords: fish; *Toxotes chatareus*; acuity; colour vision; opsin; chromophore

1. INTRODUCTION

Visual ecology is the study of how visual systems have adapted to their visual tasks and light environments. In aquatic systems, light environments are heavily dependent on the optical properties of water and suspended particulate matter therein, which greatly affect the distance over which vision may be useful and alter the transmission of light at different wavelengths. When fishes from different habitats are compared, there is a general correlation between the spectral position of their cone pigments and the background spectral environment, with the correlation being strongest for double cones (Bowmaker *et al.* 1994; Lythgoe *et al.* 1994; McDonald & Hawryshyn 1995; Cummings & Partridge 2001). The adaptive value of spectral tuning has been further supported by examples where visual pigments change within a single species as they move between habitats (Carlisle & Denton 1959; Wood *et al.* 1992; Shand 1993; Loew *et al.* 2002; Temple *et al.* 2008b). But what about eyes that simultaneously look at two different spectral environments?

It has been observed in some fishes (Levine *et al.* 1979; Bridges 1982; Burkhardt *et al.* 1983; Takechi & Kawamura 2005; Temple *et al.* 2006), amphibians (Reuter *et al.* 1971), lizards (Bowmaker *et al.* 2005),

birds (Bowmaker 1977; Hart *et al.* 2006) and mammals (Ferree & Rand 1919; Szel *et al.* 1992; Röhlich *et al.* 1994; Panorgias *et al.* 2009) that different parts of the retina employ different visual pigments or proportions thereof, or spectral filters that differentially tune spectral sensitivity. This poorly understood phenomenon—which we will hereafter refer to as ‘intraretinal differential spectral tuning’ a form of intraretinal variability—is potentially a powerful tool for explaining the role of specific sets of visual pigments within an animal eye. However, explaining the functional significance of different sets of visual pigments within any eye has proved challenging because we often know too little about the visual tasks and selective pressures that drive eye evolution. This is exemplified by our own eyes, in which proportions of the three cone types (red, green and blue) in the retina differ with eccentricity for which there, is as yet, no clear explanation (Ferree & Rand 1919; Szel *et al.* 1996; Mullen & Kingdom 2002). We demonstrate that archerfish also employ intraretinal differential spectral tuning, and we propose a model to explain the spectral position of the visual pigments in different parts of the eye by correlating the spectral sensitivity with the visual tasks associated with each visual axis.

The hunting tactic of archerfish (family Toxotidae), which spit jets of water to knock terrestrial prey down to the water's surface, was first brought to the attention of Western science nearly 250 years ago in *Philosophical Transactions of the Royal Society London* (Schlosser 1764). Since then research on archerfish has focused on

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the biomechanics of spitting (Myers 1952; L\"{u}ling 1958; Milburn *et al.* 1976; Elshoud & Koomen 1985), and spitting accuracy (Herald 1956; Bekoff & Dorr 1976; Timmermans 2000, 2001), particularly in light of the distortions that occur at the air–water interface owing to refraction (Dill 1977; Barta & Horvath 2003; Schuster *et al.* 2004; Temple 2007). Recently, archerfish have become a model for investigating learning (Timmermans & Vossen 2000; Schuster *et al.* 2004, 2006), as well as neural coding and circuitry in the visual system (Segev *et al.* 2007). Yet, surprisingly little is known about archerfish basic biology (Simon & Mazlan 2008; Simon *et al.* 2008, 2009) let alone their vision (L\"{u}ling 1956, 1958; Braekvelt 1985a,b; Plotkin *et al.* 2008), which is central to foraging and predator evasion both above and below the water's surface. We report on two basic parameters of archerfish vision; visual pigments and spatial resolving power, which were found to be tuned to life at the interface between air and water with specializations for spitting at targets above the water's surface.

2. MATERIAL AND METHODS

(a) Microspectrophotometry

Ten wild-caught large-scale archerfish (*Toxotes chatareus* Hamilton 1822) were obtained from a local supplier. Total length ranged from 5.5 to 10 cm. Fish were adapted to dark for at least 1 h prior to being killed with an overdose euganol (100 mg l⁻¹), followed by cervical transection under dim red light. Preparation of retinal tissue for microspectrophotometry (MSP) has been described in detail elsewhere (Hart 2004). Retinal samples (1–2 mm²) were taken from several locations around the retina (central; dorsal, ventral, temporal and nasal periphery; and intermediate locations along the ventral periphery). The retina was oriented using the prominent falciform process that projects from the optic nerve head ventro-nasally.

Photoreceptor outer segment absorbance was measured from 330 to 800 nm using a single-beam, wavelength scanning microspectrophotometer (Hart 2004). A sample scan was made from each outer segment and a baseline scan made from a tissue-free area of the preparation adjacent to the photoreceptor. A prebleach absorbance spectrum was calculated from the sample and baseline measurements. Following a 1 min full spectrum (white light) bleach of the outer segment, sample and baseline scans were repeated to obtain a postbleach spectrum that was subtracted from the prebleach spectrum to give a bleaching difference spectrum. Absorbance spectra were analysed following the procedures described previously (Hart 2002).

A custom computer program designed (by N.S.H.) to iteratively fit mixed opsin and mixed A₁/A₂ template models to MSP data was used to fit many absorbance curves, particularly single cones isolated from the ventro-nasal retina, that were not fit well by standard templates (full-width at half-maximum bandwidth wider than templates) derived for vitamin A₁- and vitamin A₂-based visual pigments (see below). Free parameters were: $\lambda_{\max}$ of the two opsins in their A₁ state; ratio of two opsins; and ratio of A₁:A₂. The model that gave the smallest least squares difference between the real data (running average of the raw absorbance curve) and the model (between 400 and 700 nm) was selected. All curves were fitted well with these mixed templates. However, the presence of three or more

opsins in any one cone and the influence of stable photoproducts on spectral shape cannot be excluded using this approach.

One-way analysis of variance was used to compare $\lambda_{\max}$ values of photoreceptors from different retinal areas. Tukey HSD post hoc tests were used to identify differences among groups. Independent sample *t*-tests were used to compare $\lambda_{\max}$ values of the two outer segment members of double cones. Statistical analyses were performed using SPSS (v. 12.0, SPSS Inc., IL, USA).

(b) Measurements of background spectral light environment

Spectral quality of environmental light was measured in the Laura River, Cape York, Queensland, Australia (15°34'44.23 S; 144°27'25.31 E), where archerfish were found, at midday on a sunny cloudless day. Spectral radiance was measured from 300 to 850 nm with a portable fibre-optic spectrometer (S2000, Ocean Optics Inc., FL, USA). The fibre diameter was 50 μ m with an acceptance angle of 22.6° or approximately 0.126 steradians. Spectral radiance was recorded as relative photon counts and spectra were normalized for direct comparison of the relative contribution in different areas of the spectrum.

(c) Measuring ocular media spectral transmittance

A small hole (2 mm diameter) was cut in the back of the eye, opposite the pupil and all layers from sclera to retina were removed, leaving cornea, lens and vitreous humor. Spectral transmittance was measured by projecting full spectrum light (250–850 nm; PX-2 pulsed xenon light source, Ocean Optics Inc.) with a 200 μ m fibre optic through the eye and recording transmitted light on the other side with a 1000 μ m fibre optic connected to a spectrometer (USB4000, Ocean Optics Inc.). Lens and cornea were also measured separately.

(d) Estimating spectral sensitivity

The corrected spectral sensitivity of each photoreceptor 'type' was calculated (see Hart & Vorobyev 2005) using the visual pigment absorbance templates of Govardovskii *et al.* (2000), a specific (decadic) absorbance of 0.0125 μ m⁻¹ and a path length estimated from mean photoreceptor lengths for each cell type from each part of the retina measured onscreen during MSP. The on-axis absorbance calculated in this manner was converted to absorptance and multiplied by the spectral transmittance of the ocular media.

$$\text{Corrected absorptance} = \sum_{800}^{350} A \times A_s \times l \times T_{\text{om}}$$

(e) Retinal topography of photoreceptor and ganglion cells

Enucleated eyes were fixed in 4 per cent paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1.5 h. Retinae were dissected free of the eye cup and as much of the retinal pigment epithelium (RPE) was removed as possible without causing damage to the underlying photoreceptor layer. In all cases, it was not possible to remove RPE from an area in the ventral hemisphere (area of high cone density, see §3). To enable counting in this retinal area, the RPE was cleared by bleaching in 3 per cent hydrogen peroxide in 0.1 M phosphate buffer (pH adjusted to 11.95). Peripheral slits were cut to flatten the retina. Retinas were whole-mounted

photoreceptor side upwards for photoreceptor counts. A plastic spacer of approximately 150 μm thickness was placed between slide and coverslip to maintain photoreceptor orientation and spacing. Retinas were cleared with a solution of 50 per cent glycerol and 50 per cent phosphate buffer. Coverslip and spacer were sealed to the slide with nail polish to prevent desiccation. To enable direct comparisons of photoreceptor and ganglion cell densities in the same eye, wholemounts were flipped over for ganglion cell counting (as described in Litherland & Collin 2008) and stained (following Oliveira *et al.* 2006).

Photoreceptor and ganglion cell counting was performed on a Zeiss Axioplan II compound microscope fitted with an x-y-z motorized stage (BioPrecision, LUDL Electronic Products Inc., NY, USA). A randomly assigned sampling grid was created using STEREO INVESTIGATOR 6 (MicroBrightField Inc., Vermont, USA) software, which also controlled stage movement. Counts were made at 0.5 mm intervals with a counting frame of 75 μm^2 , providing about 200 sample locations across the retina. High-frequency sampling was done in the ventral retina to delineate the areas of interest. Double/twin cones were counted as single units. Ganglion and amacrine cells were differentiated from glial cells by their cytoplasmic staining. Ganglion and amacrine cells were not differentiated, as elsewhere (Oliveira *et al.* 2006), and therefore these results represent the upper limits of spatial resolving power for fish of this size of this species.

Topographic maps of cone photoreceptor and ganglion cells were created by drawing isodensity contour lines between areas with similar densities as described previously (Collin & Pettigrew 1988). Spatial resolving power was estimated using the equations provided in Collin & Pettigrew (1989).

3. RESULTS

(a) Microspectrophotometry

Spectral absorbance was measured from 303 photoreceptors sampled from several locations (see §2a) across the retinæ of six archerfish (table 1). The λ_{max} values of single and double cones measured differed markedly across the retina and fit one of the three patterns (figure 1 and electronic supplementary material, figure S1) coinciding with three broad retinal areas: dorsal, ventro-temporal and ventro-nasal. Single cones and rods did not differ significantly in λ_{max} between dorsal and ventro-temporal areas ($p \geq 0.05$ for both), but did differ significantly between both of these areas and the ventro-nasal area ($p < 0.001$ for both). Double cone λ_{max} values differed significantly among all three retinal areas ($p < 0.001$). The λ_{max} values of both outer segment members were significantly different from one another in the ventro-temporal area ($p < 0.001$) and ventro-nasal area ($p = 0.01$), they did not differ in the dorsal area ($p = 0.063$). The differences in the ventro-nasal and dorsal areas were only slightly greater than the estimated measurement error of ± 3 nm (table 1). Maximum absorbance of rods ranged from 499 to 527 nm (figure 1 and table 1), consistent with a change in chromophore (vitamin A₁/A₂) ratio within a single opsin (Temple *et al.* 2008a).

(b) Optical media transmittance

The spectral transmission of light reaching the retina through the optical media (cornea, lens and vitreous

Table 1. Microspectrophotometric data from different areas of the archerfish (*Toxotes chatareus*) retina. OS, outer segment; HBW, half maximum width; abs., absorbance.

retinal location	photoreceptor class	number of cells	mean OS		mean cell base		minimum		maximum		mean HBW		mean	
			length (μm)	length (μm)	diameter (μm)	λ_{max} (nm)	s.d.	λ_{max} (nm)	λ_{max} (nm)	(nm)	s.d.	max abs.	s.d.	
dorsal	rods	36	25.8	35.0	1.7	504.3	2.3	499	508	101.4	5.1	0.0230	0.0081	
	single cones	29	9.3	20.0	3.0	454.0	8.5	441	473	99.9	13.7	0.0240	0.0086	
	double cones short	27	7.1	13.0	2.3	567.7	13.6	522	580	129.1	11.0	0.0245	0.0085	
	double cones long	30	7.3	13.0	2.3	573.5	9.7	539	584	128.6	11.2	0.0292	0.0308	
	rods	32	21.8	35.0	1.4	507.2	5.1	500	522	105.0	5.8	0.0184	0.0051	
	single cones	20	6.2	10.0	2.4	453.3	7.1	442	467	100.7	10.5	0.0223	0.0077	
	double cones short	26	6.7	17.0	1.9	534.9	12.9	516	560	125.2	19.4	0.0222	0.0055	
	double cones long	26	6.3	17.0	1.9	564.5	16.2	520	584	132.8	11.9	0.0216	0.0075	
	rods	22	26.1	35.0	1.3	515.2	7.2	502	527	120.9	19.3	0.0155	0.0050	
ventro-nasal	single cones	17	7.1	14.0	1.9	502.4	14.2	477	521	151.1	22.6	0.0133	0.0051	
	double cones short	18	12.2	18.0	1.9	617.3	4.9	606	623	158.3	13.2	0.0182	0.0034	
	double cones long	20	10.4	18.0	1.8	621.7	5.0	607	628	171.8	35.5	0.0185	0.0062

Figure 1. Mean maximum absorbance ($\lambda_{\max} \pm \text{s.d.}$) of archerfish (*Toxotes chatareus*) photoreceptors fit three basic patterns coinciding with three retinal areas, measured using microspectrophotometry. Grey crosses, rods; circles, single cones; triangles, each outer segment member of the double cones (ventro-temporally the $\lambda_{\max}$ values of the two outer segment members differed significantly ($p < 0.001$) and are connected by a dashed line to indicate that they are one functional unit).

Figure 2. Normalized corrected absorbance curves (see text) for single cones (dashed lines) and double cones (solid and dotted lines) measured from three areas of the archerfish (*Toxotes chatareus*) retina, compared with normalized spectral radiance (grey filled curves) for the respective field of view (measured in the natural environment; Laura River, Cape York, Queensland, Australia). The lack of dotted lines in plots of dorsal and ventro-nasal areas reflects the similarity in $\lambda_{\max}$ values of the two outer segment members of the double cones in these retinal areas; a single line was used to reduce crowding.

humor) was primarily limited by the transmission properties of the lens. Transmittance through cornea, lens and vitreous humor combined was above 80 per cent from 480 to 750 nm. Transmission was decreased to 50 per cent (λ_{T50} or T_{50}) at 414 nm in the short wavelengths and at 843 nm in the long wavelengths (electronic supplementary material, figure S2).

(c) Comparing spectral tuning to background spectral light environment

Corrected spectral absorbance of cone photoreceptors (see §2c) from the three retinal areas were normalized to the spectral radiance measured in the natural environment along the optical axes of these three retinal areas

(figure 2). Upwelling spectral radiance, projecting to the dorsal retina, showed marked attenuation at short wavelengths compared with the radiance of downwelling skylight projecting onto the ventro-temporal and ventro-nasal retinal areas (figure 2).

Dorsally, twin cones were closely matched to upwelling spectral radiance, while single cones were offset to shorter wavelengths. Ventro-temporally, all cone types were equally well matched/offset to the downwelling radiance of skylight, while ventro-nasally, it was the single cones that were tuned to longer wavelengths, where they matched the downwelling skylight and the twin cones were offset to longer wavelengths (figure 2).

(d) Retinal topography

Cone photoreceptor densities ranged from 5700 cells mm^{-2} dorsally to 51 300 cells mm^{-2} ventrally, and patterns of isodensity contours were consistent in all three fish. Average cone densities dorsally were approximately 16 000 cells mm^{-2} increasing towards the periphery. The ventral retina was characterized by an area of high density (greater than 22 500 cells mm^{-2}) that was aligned with the projection of Snell's window onto the retina. Within this area, there were three smaller areas of higher densities: one temporally and one nasally each with average cone densities of approximately 41 000 cells mm^{-2} ; and one ventrally aligned with the preferred spitting angle, where cone densities reached greater than 50 000 cells mm^{-2} (figure 3).

Ganglion cell density patterns were similar to that of cones (figure 3). Dorsally, ganglion cell densities averaged 5000 cells mm^{-2} increasing towards the periphery. The ventral retina was characterized by an area of high density (greater than 20 000 cells mm^{-2}) that was also aligned with the projection of Snell's window onto the retina. Highest ganglion cell densities were found ventro-temporally and were aligned with the preferred spitting angle. Here, ganglion cell density was greater than 50 000 cells mm^{-2} ; providing nearly a 1 : 1 convergence ratio with the photoreceptors (figure 3). Anatomical spatial resolving power was highest at 6.9 cycles per degree for the up-forward visual axis and lowest at 2.2 cycles per degree for downwards visual axis.

4. DISCUSSION

(a) Intraretinal differential spectral tuning

The compliment of visual pigments in the archerfish differed in three retinal areas associated with three different

Figure 3. Topographic distribution of cones (left inset) and ganglion cells (right inset) of the archerfish (*Toxotes chatareus*) correctly oriented and overlaid on an image of an archerfish spitting. Cell densities are reported as cells mm^{-2} . Red line demarcates the line of sight to the target, note the bending of light as it passes through the air–water interface owing to differences in refractive indices of air and water.

visual axes, each tuned to a different spectral light environment or visual task. In the dorsal retina, archerfish possessed single and twin cones with peak spectral sensitivities at 454 nm and 570 nm, respectively. Compared with the upwelling background light environment (figure 2), this pair of visual pigments provides one matched and one offset detector, maximizing contrast of both light and dark targets approaching from below (Lythgoe 1966; McFarland & Munz 1975). Under this scenario, dark (non-reflecting) targets will be high-contrast silhouettes to the twin cones (luminosity detectors) spectrally matched to the background, but single cones will play little role as they are relatively insensitive to the background (figure 2). Conversely, light (reflecting) targets will require output from both cell types to be compared, which is the basis of chromaticity detection. For twin cones, light targets would appear slightly brighter than the background as a result of greater intensity of downwelling to upwelling light, while for single cones, light targets will appear bright against a dark background as they reflect full spectrum downwelling light rich in short wavelengths (having travelled through less water). This pair of visual pigments, if compared in an inhibitory fashion, would be ideally suited for discriminating shades of brown (Lythgoe & Partridge 1989), the colour of upwelling light in the mangroves and rivers where archerfish are found. Similar combinations of matched and offset pigments have been

demonstrated to optimize contrast detection in numerous habitats (McFarland & Munz 1975; Lythgoe *et al.* 1994; Cummings 2004) and in all cases double cones have been found to be matched to the background.

In the ventro-nasal retina, which looks up and behind, archerfish were found to possess single and twin cones at 502 nm and 620 nm, respectively. When compared with the spectral background of downwelling skylight, the single cones are well matched, suitable for detecting dark silhouettes against the bright sky, and twin cones are offset and may function to detect targets reflecting long wavelength-shifted upwelling light. The observation that single cones in the ventro-nasal retina of archerfish are matched to the spectral background is evidence against the proposal that double cones are somehow specially suited for the role of being matched to the background light environment (Loew & Lythgoe 1978). We suggest that double cone spectral matching may be limited to dim, poorly lit, environments where the signal to noise ratio is low. In fact, several other fishes, that swim predominantly just below the water's surface, have been found to possess single cones with peak sensitivities between 490 and 510 nm (Loew & Lythgoe 1978; Levine & MacNichol 1979; Britt *et al.* 2001; Allison *et al.* 2004), which is a close match to skylight. And among those, several have been shown to possess intraretinal variability (Levine *et al.* 1979) including zebrafish (*Danio rerio*; Takechi & Kawamura 2005), suggesting that intraretinal variability may be common among fishes living near the water's surface.

In the ventro-temporal retina, archerfish were found to possess single cones at 453 nm and double cones with one member at 535 and the other member at 565 nm. If used for chromatic discrimination, this part of the retina would provide archerfish with trichromatic colour vision in the visual axis used for detecting prey items against a background of overhanging foliage, much like the human trichromatic visual system (blue = 420 nm; green = 533 nm; and red = 564 nm), which is thought to be adapted to the same task (Osorio & Vorobyev 1996).

Despite the fact that nearly every species with multiple visual pigments, that has been tested behaviourally, has been found to possess the capacity for colour vision, there remains resistance to presuming that a species with multiple cone pigments possesses colour vision (Kelber *et al.* 2003). For many fishes, this problem is exacerbated by uncertainty about the role of double cones in colour vision (Douglas & Hawryshyn 1990; Northmore *et al.* 2007). Double cones are thought to act as luminosity detectors (Yoshizawa 1994; Osorio & Vorobyev 2005), and when tuned to the background are proposed to improve spatial resolution of movement detection (Lyall 1957; Wagner 1990), though they are also proposed to play a role in polarization sensitivity (Cameron & Pugh 1991; Hawryshyn 2000). While most fishes possess double cones, few have been tested behaviourally for the possession of colour vision and, of those, the goldfish (*Carassius auratus*) is the most well established model. It is thought that goldfish use UV, short-, middle- and long-wavelength sensitive single cones to mediate colour vision (Neumeyer 2003) but not their double cones. However, recent evidence from our laboratory has found behavioural evidence for trichromacy in a fish with only one single cone sensitive to

short wavelengths and a double cone sensitive to middle- and long-wavelengths (Pignatelli *et al.* 2010).

The proximate cause of intraretinal differential spectral tuning in archerfish is a combination of changes in visual pigment chromophore (vitamin A₁/A₂) ratio and differential opsin expression. Although opsin sequences and *in situ* hybridization are currently not available, we have two lines of evidence to support changes in A₁/A₂ ratio and differential opsin expression. Firstly, the $\lambda_{\max}$ of rods varied from 499 to 527 nm, which fits models predicting the shift in $\lambda_{\max}$ expected for a change in chromophore ratio combined with a single opsin. Both members of the double cone in the ventro-nasal retina had $\lambda_{\max}$ values in excess of 575 nm, only possible with A₂-based visual pigments (reviewed in Temple *et al.* 2008a). Secondly, we were unable to fit template curves (A₁, A₂ or mixed A₁ and A₂) to many absorbance spectra particularly those of single cones from the ventro-nasal retina. Using our custom-designed computer program, we were able to iteratively fit these curves with mixed templates that included a variable ratio of A₁/A₂ and two different opsins. A molecular approach is underway to determine the number of opsins and their subtypes expressed in *T. chatareus*. It will be interesting to compare the archerfish opsin repertoire to that of *Anableps anableps*, another fish that lives at, and sees through, the interface between air and water, and that was recently found to express 10 retinal opsins (Owens *et al.* 2009).

(b) Spatial resolving power

Spatial resolving power (6.9 cycles per degree) in archerfish is among the highest reported for freshwater fishes (Douglas & Hawryshyn 1990) and is comparable to that found in some terrestrial animals (6 cycles per degree in rabbit and rhinoceros; 7 cycles per degree in agouti (table 1 in Pettigrew & Manger 2008)). Cone and ganglion cell densities were highest in ventro-temporal retina, where a convergence ratio of 1:1 was revealed, as occurs in human fovea. Using frame grabs from high-definition video recordings and close-up still images of eyes during spitting, we found that this area was aligned with the image of the target when spitting. An alignment that persist across most spitting angles as archerfish eyes rotate in the orbit such that the angle relative to the target remains constant despite dramatic changes in body angle (Timmermans & Souren 2004; Segev *et al.* 2007). Visual resolution in the ventro-temporal retina would allow archerfish to resolve two objects separated by 2 mm at a distance of 550 mm (moderate spitting distance for a 10 cm archerfish).

Two additional areas of high cone density were found in the ventral retina, one temporally and one ventrally. These were aligned with the edges of the projected image of Snell's window onto the retina. Higher spatial resolution in these areas provides compensation for increased compression of aerial images occurring at the borders of Snell's window. The fish would also be able to align the temporal area with the mouth axis, making it functional in the final approach of downed and aquatic prey.

In general, the order of magnitude of higher cone density in the ventral retina matches not only the greater distances over which vision is useful in air versus water, but also matches the decreased need for increased

photon catch in the ventral retina that necessarily receives images of much greater intensity.

Compared with previous reports, our measure of highest ganglion cell density is an order of magnitude greater (Lüling 1956; Segev *et al.* 2007), which can be accounted for by our efforts to maintain retinal integrity out to the periphery where densities were highest, and our use of bleaching to clear the retinal pigment epithelium, which otherwise obviates cell counting in the ventral periphery.

5. CONCLUSIONS

Archerfish were found to have intraretinal variability in visual pigments; a phenomenon that evidence would suggest is widespread among vertebrates (Denton *et al.* 1971; Reuter 1972; Levine *et al.* 1979; Huang 1989; Bowmaker 1991; Szel *et al.* 1996; Helvik *et al.* 2001; Takechi & Kawamura 2005; Temple *et al.* 2006). The implications of differential visual pigment use across the retina of this and other fishes are relevant to studies that have predicted spectral sensitivity of the eye as a whole based on MSP data that may have been collected from limited areas within the retina. Intraretinal differences in visual pigments may account for differences in reported spectral sensitivities of various fishes.

What remains to be tested in archerfish, and other species with intraretinal differences in visual pigments, is precisely how this phenomenon affects spectral sensitivity and hue discrimination in different visual axes. For this, archerfish are an excellent model as they are easily trained to spit at coloured targets under operant conditioning paradigms, and experiments are underway to address this question.

All procedures were approved by The University of Queensland Animal Ethics Committee (AEC no. SBMS/541/08).

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REFERENCES

- Allison, W. T., Haimberger, T. J., Hawryshyn, C. W. & Temple, S. E. 2004 Visual pigment composition in zebrafish: evidence for a rhodopsin-porphyrin interchange system. *Vis. Neurosci.* **21**, 945–952.
- Barta, A. & Horvath, G. 2003 Underwater binocular imaging of aerial objects versus the position of eyes relative to the flat water surface. *J. Opt. Soc. Am.* **20**, 2370–2377. (doi:10.1364/JOSAA.20.002370)
- Bekoff, M. & Dorr, R. 1976 Predation by 'shooting' in archer fish, *Toxotes jaculatrix*: accuracy and sequences. *Bull. Psychonom. Soc.* **7**, 167–168.
- Bowmaker, J. K. 1977 The visual pigments, oil droplets and spectral sensitivity of the pigeon. *Vision Res.* **17**, 1129–1138. (doi:10.1016/0042-6989(77)90147-X)
- Bowmaker, J. K. 1991 The evolution of vertebrate visual pigments and photoreceptors. In *Evolution of the eye and visual system*, vol. 2 (eds J. R. Cronly-Dillon & R. L. Gregory), pp. 63–81. Boca Raton, FL: CRC Press, Inc.

- Bowmaker, J. K., Govardovskii, V. I., Shukolyukov, S. A., Zueva, L. V., Hunt, D. M., Sideleva, V. G. & Smirnova, O. G. 1994 Visual pigments and the photic environment: the cottoid fish of Lake Baikal. *Vision Res.* **34**, 591–605. (doi:10.1016/0042-6989(94)90015-9)
- Bowmaker, J. K., Loew, E. R. & Ott, M. 2005 The cone photoreceptors and visual pigments of chameleons. *J. Comp. Physiol. A* **191**, 925–932. (doi:10.1007/s00359-005-0014-4)
- Braekevelt, C. R. 1985a Fine structure of the retinal pigment epithelial region of the archerfish (*Toxotes jaculatrix*). *Ophthalm. Res.* **17**, 221–229. (doi:10.1159/000265376)
- Braekevelt, C. R. 1985b Photoreceptor fine structure in the archerfish (*Toxotes jaculatrix*). *Am. J. Anat.* **173**, 89–98. (doi:10.1002/aja.1001730203)
- Bridges, C. D. B. 1982 Porphyropsin in retina of four-eyed fish *Anableps anableps*. *Nature* **300**, 384. (doi:10.1038/300384a0)
- Britt, L. L., Loew, E. R. & McFarland, W. N. 2001 Visual pigments in the early life stages of Pacific northwest marine fishes. *J. Exp. Biol.* **204**, 2581–2587.
- Burkhardt, D. A., Gottesman, J., Levine, J. S. & MacNichol Jr, E. F. 1983 Cellular mechanisms for color-coding in holostean retinas and the evolution of color vision. *Vision Res.* **23**, 1031–1041. (doi:10.1016/0042-6989(83)90014-7)
- Cameron, D. A. & Pugh, E. N. 1991 Double cones as a basis for a new type of polarization vision in vertebrates. *Nature* **353**, 161–164. (doi:10.1038/353161a0)
- Carlisle, D. B. & Denton, E. J. 1959 On the metamorphosis of the visual pigments of *Anguilla anguilla* (L.). *J. Mar. Biol. Assoc. UK* **38**, 97–102. (doi:10.1017/S0025315400015629)
- Collin, S. P. & Pettigrew, J. D. 1988 Retinal topography in reef teleosts 1: some species with well-developed areae but poorly-developed streaks. *Brain Behav. Evol.* **31**, 269–282. (doi:10.1159/000116594)
- Collin, S. P. & Pettigrew, J. D. 1989 Quantitative comparison of the limits on visual spatial resolution set by the ganglion cell layer in twelve species of reef teleosts. *Brain Behav. Evol.* **34**, 184–192. (doi:10.1159/000116504)
- Cummings, M. E. 2004 Modelling divergence in luminance and chromatic detection performance across measured divergence in surfperch (Embiotocidae) habitats. *Vision Res.* **44**, 1127–1145. (doi:10.1016/j.visres.2003.12.013)
- Cummings, M. E. & Partridge, J. C. 2001 Visual pigments and optical habitats of surfperch (Embiotocidae) in the California kelp forest. *J. Comp. Physiol. A* **187**, 875–889.
- Denton, E. J., Muntz, W. R. & Northmore, D. P. 1971 The distribution of visual pigment within the retina in two teleosts. *J. Mar. Biol. Assoc. UK* **51**, 905–915. (doi:10.1017/S002531540001804X)
- Dill, L. M. 1977 Refraction and the spitting behavior of archerfish (*Toxotes chatareus*). *Behav. Ecol. Sociobiol.* **2**, 169–184.
- Douglas, R. H. & Hawryshyn, C. W. 1990 *Behavioural studies of fish vision: an analysis of visual capabilities. The visual system of fish*. London, UK: Chapman & Hall.
- Elshoud, G. C. A. & Koomen, P. 1985 A biomechanical analysis of spitting in archer fishes (Pisces, Perciformes, Toxidae). *Zoomorphology* **105**, 240–252. (doi:10.1007/BF00311967)
- Ferree, C. E. & Rand, G. 1919 Chromatic thresholds of sensation from center to periphery of the retina and their bearing on color theory. *Psychol. Rev.* **26**, 16–41. (doi:10.1037/h0071940)
- Govardovskii, V. I., Fyhrquist, N., Reuter, T., Kuzmin, D. G. & Donner, K. 2000 In search of the visual pigment template. *Vis. Neurosci.* **17**, 509–528. (doi:10.1017/S0952523800174036)
- Hart, N. S. 2002 Vision in the peafowl (Aves: *Pavo cristatus*). *J. Exp. Biol.* **205**, 3925–3935.
- Hart, N. S. 2004 Microspectrophotometry of visual pigments and oil droplets in a marine bird, the wedge-tailed shearwater *Puffinus pacificus*: topographic variations in photoreceptor spectral characteristics. *J. Exp. Biol.* **207**, 1229–1240. (doi:10.1242/jeb.00857)
- Hart, N. S. & Vorobyev, M. 2005 Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. *J. Comp. Physiol. A* **191**, 381–392. (doi:10.1007/s00359-004-0595-3)
- Hart, N. S., Lisney, T. J. & Collin, S. P. 2006 Cone photoreceptor oil droplet pigmentation is affected by ambient light intensity. *J. Exp. Biol.* **209**, 4776–4787. (doi:10.1242/jeb.02568)
- Hawryshyn, C. W. 2000 Ultraviolet polarization vision in fishes: possible mechanisms for coding e-vector. *Phil. Trans. R. Soc. Lond. B* **355**, 1187–1190. (doi:10.1098/rstb.2000.0664)
- Helvik, J. V., Drivenes, O., Naess, T. H., Fjose, A. & Seo, H. C. 2001 Molecular cloning and characterization of five opsin genes from the marine flatfish Atlantic halibut (*Hippoglossus hippoglossus*). *Vis. Neurosci.* **18**, 767–780.
- Herald, E. S. 1956 How accurate is the archer fish? *Pacific Discov.* **9**, 12–13.
- Huang, Q. 1989 Intraretinal distribution of cone pigments in black porgy, *Acanthopagrus schlegelii*. *Bull. Inst. Zool. Acad. Sin.* **28**, 175–182.
- Kelber, A., Vorobyev, M. & Osorio, D. 2003 Animal colour vision—behavioural tests and physiological concepts. *Biol. Rev. Camb. Phil. Soc.* **78**, 81–118.
- Levine, J. S. & MacNichol, E. F. 1979 Visual pigments in teleost fishes: effects of habitat, microhabitat, and behavior on visual system evolution. *Sens. Process.* **3**, 95–131.
- Levine, J. S., MacNichol Jr, E. F., Kraft, T. & Collins, B. A. 1979 Intraretinal distribution of cone pigments in certain teleost fishes. *Science* **204**, 523–526. (doi:10.1126/science.432658)
- Litherland, L. & Collin, S. P. 2008 Comparative visual function in elasmobranchs: spatial arrangement and ecological correlates of photoreceptor and ganglion cell distributions. *Vis. Neurosci.* **25**, 549–561. (doi:10.1017/S0952523808080693)
- Loew, E. R. & Lythgoe, J. N. 1978 The ecology of cone pigments in teleost fish. *Vision Res.* **18**, 715–722. (doi:10.1016/0042-6989(78)90150-5)
- Loew, E. R., McFarland, W. N. & Margulies, D. 2002 Developmental changes in the visual pigments of the yellowfin tuna, *Thunnus albacares*. *Mar. Freshw. Behav. Physiol.* **35**, 235–246. (doi:10.1080/1023624021000003826)
- Lüling, K. H. 1956 Über das Leuchtvermögen juveniler *Toxotes jaculatrix* (Pallas 1766). *Zool. Beiträge Bonn* **7**, 58.
- Lüling, K. H. 1958 Morphologisch-anatomische und histologische Untersuchungen am Auge des Schützenfisches *Toxotes jaculatrix* (Pallas 1766) (Toxotidae), nebst Bemerkungen zum Spuckgehaben. *Zeitschrift für Morphologie und Ökologie der Tiere* **47**, 529–610. (doi:10.1007/BF00389870)
- Lyall, A. H. 1957 Cone arrangements in teleost retinae. *Q. J. Microsc. Sci.* **98**, 189.
- Lythgoe, J. N. 1966 Visual pigments and underwater vision. In *Light as an ecological factor*, vol. 6 (eds R. Bainbridge, C. C. Evans & D. Rackham), pp. 375–391. Oxford, UK: Blackwell.
- Lythgoe, J. N. & Partridge, J. C. 1989 Visual pigments and the acquisition of visual information. *J. Exp. Biol.* **146**, 1–20.
- Lythgoe, J. N., Muntz, W. R. A., Partridge, J. C., Shand, J. & Williams, D. M. 1994 The ecology of the visual pigments

- of snappers (Lutjanidae) on the Great Barrier Reef. *J. Comp. Physiol. A* **174**, 461–467.
- McDonald, C. G. & Hawryshyn, C. W. 1995 Intraspecific variation of spectral sensitivity in threespine stickleback (*Gasterosteus aculeatus*) from different photic regimes. *J. Comp. Physiol. A* **176**, 255–260.
- McFarland, W. N. & Munz, F. W. 1975 Part III. The evolution of photopic visual pigments in fishes. *Vision Res.* **15**, 1071–1080. (doi:10.1016/0042-6989(75)90003-6)
- Milburn, O., Alexander, R. & McN 1976 The performance of the muscles involved in spitting by the archerfish *Toxotes*. *J. Zool.* **180**, 243–251. (doi:10.1111/j.1469-7998.1976.tb04676.x)
- Mullen, K. T. & Kingdom, F. A. A. 2002 Differential distributions of red–green and blue–yellow cone opponency across the visual field. *Vis. Neurosci.* **19**, 109–118.
- Myers, G. S. 1952 How the shooting apparatus of the archer fish was discovered. *Aquarium J.* **23**, 210–214.
- Neumeyer, C. 2003 Color vision in fishes and its neural basis. In *Sensory processing in aquatic environments* (eds S. P. Collin & N. J. Marshall), pp. 223–235. New York, NY: Springer.
- Northmore, D. P. M., Oh, D. J. & Celenza, M. A. 2007 Acuity and contrast sensitivity of the bluegill sunfish and how they change during optic nerve regeneration. *Vis. Neurosci.* **24**, 319–331.
- Oliveira, F. G. *et al.* 2006 Topographic analysis of the ganglion cell layer in the retina of the four-eyed fish *Anableps anableps*. *Vis. Neurosci.* **23**, 879–886. (doi:10.1017/S0952523806230232)
- Osorio, D. & Vorobyev, M. 1996 Colour vision as an adaptation to frugivory in primates. *Proc. R. Soc. Lond. B* **263**, 593–599. (doi:10.1098/rspb.1996.0089)
- Osorio, D. & Vorobyev, M. 2005 Photoreceptor spectral sensitivities in terrestrial animals: adaptations for luminance and colour vision. *Proc. R. Soc. Lond. B* **272**, 1745–1752. (doi:10.1098/rspb.2005.3156)
- Owens, G. L., Windsor, D. J., Mui, J. & Taylor, J. S. 2009 A fish eye out of water: ten visual opsins in the four-eyed fish, *Anableps anableps*. *PLoS One* **4**, e5970. (doi:10.1371/journal.pone.0005970)
- Panorgias, A., Parry, N. R. A., McKeefry, D. J., Kulikowski, J. J. & Murray, I. J. 2009 Nasal-temporal differences in cone-opponency in the near peripheral retina. *Ophthalm. Physiol. Opt.* **29**, 375–381. (doi:10.1111/j.1475-1313.2009.00666.x)
- Pettigrew, J. D. & Manger, P. R. 2008 Retinal ganglion cell density of the black rhinoceros (*Diceros bicornis*): calculating visual resolution. *Vis. Neurosci.* **25**, 215–220.
- Pignatelli, V., Champ, C., Marshall, J. & Vorobyev, M. 2010 Double cones are used for colour vision discrimination in reef fish, *Rhinecanthus aculeatus*. *Biol. Lett.* **6**, 537–539. (doi:10.1098/rsbl.2009.1010)
- Plotkin, A., Paperno, E., Vasserman, G. & Segev, R. 2008 Magnetic tracking of eye motion in small, fast-moving animals. *IEEE Trans. Magn.* **44**, 4492–4495. (doi:10.1109/Tmag.2008.2002187).
- Reuter, T. 1972 Long-lived photoproducts in the retinae of the frog and the crucian-carp. In *Biochemistry and physiology of visual pigments* (ed. H. Langer), pp. 211–217. Bochum, West Germany: Springer.
- Reuter, T. E., White, R. H. & Wald, G. 1971 Rhodopsin and porphyropsin fields in the adult bullfrog retina. *J. Gen. Physiol.* **58**, 351–371. (doi:10.1085/jgp.58.4.351)
- Röhlich, P., van Veen, T. & Szel, A. 1994 Two different visual pigments in one retinal cone cell. *Neuron* **13**, 1159–1166. (doi:10.1016/0896-6273(94)90053-1)
- Schlosser, J. A. 1764 An account of a fish from Batavia called Jaculator: in a letter to Mr. Peter Collinson, F. R. S. from John Albert Schlosser, M. D. F. R. S. *Phil. Trans. R. Soc. Lond.* **54**, 89–91. (doi:10.1098/rstl.1764.0016)
- Schuster, S., Rossel, S., Schmidtman, A., Jager, I. & Poralla, J. 2004 Archer fish learn to compensate for complex optical distortions to determine the absolute size of their aerial prey. *Curr. Biol.* **14**, 1565–1568. (doi:10.1016/j.cub.2004.08.050)
- Schuster, S., Wohl, S., Griebisch, M. & Klostermeier, I. 2006 Animal cognition: how archer fish learn to down rapidly moving targets. *Curr. Biol.* **16**, 378–383. (doi:10.1016/j.cub.2005.12.037)
- Segev, R., Schneidman, E., Goodhouse, J. & Berry, M. J. 2007 Role of eye movements in the retinal code for a size discrimination task. *J. Neurophysiol.* **98**, 1380–1391. (doi:10.1152/jn.00395.2007)
- Shand, J. 1993 Changes in the spectral absorption of cone visual pigments during the settlement of the goatfish *Upeneus tragula*: the loss of red sensitivity as a benthic existence begins. *J. Comp. Physiol. A* **173**, 115–121.
- Simon, K. D. & Mazlan, A. G. 2008 Length–weight and length–length relationships of archer and puffer fish species. *The Open Fish Sci. J.* **1**, 19–22. (doi:10.2174/1874401X0801010019)
- Simon, K. D., Mazlan, A. G., Cob, Z. C., Samat, A. & Arshad, A. 2008 Age determination of archer fishes (*Toxotes jaculator* and *Toxotes chatareus*) inhabiting Malaysian estuaries. *J. Biol. Sci.* **8**, 1096–1099. (doi:10.3923/jbs.2008.1096.1099)
- Simon, K. D., Bakar, Y., Samat, A., Zaidi, C. C., Aziz, A. & Mazlan, A. G. 2009 Population growth, trophic level, and reproductive biology of two congeneric archer fishes (*Toxotes chatareus*, Hamilton 1822 and *Toxotes jaculator*, Pallas 1767) inhabiting Malaysian coastal waters. *J. Zhejiang Univ.-Sci. B* **10**, 902–911. (doi:10.1631/jzus.B0920173)
- Szel, A., Rohlich, P., Caffè, A. R., Juliusson, B., Aguirre, G. & Vanveen, T. 1992 Unique topographic separation of two spectral classes of cones in the mouse retina. *J. Comp. Neurol.* **325**, 327–342. (doi:10.1002/cne.903250302)
- Szel, A., Rohlich, P., Caffè, A. R. & vanVeen, T. 1996 Distribution of cone photoreceptors in the mammalian retina. *Microsc. Res. Tech.* **35**, 445–462. (doi:10.1002/(SICI)1097-0029(19961215)35:6<445::AID-JEMT4>3.0.CO;2-H)
- Takechi, M. & Kawamura, S. 2005 Temporal and spatial changes in the expression pattern of multiple red and green subtype opsin genes during zebrafish development. *J. Exp. Biol.* **208**, 1337–1345. (doi:10.1242/jeb.01532)
- Temple, S. E. 2007 Effect of salinity on the refractive index of water: considerations for archer fish aerial vision. *J. Fish Biol.* **70**, 1626–1629.
- Temple, S. E., Plate, E. M., Ramsden, S., Haimberger, T. J., Roth, W. M. & Hawryshyn, C. W. 2006 Seasonal cycle in vitamin A₁/A₂-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*). *J. Comp. Physiol. A* **192**, 301–313. (doi:10.1007/s00359-005-0068-3)
- Temple, S. E., Ramsden, S. D., Haimberger, T. J., Veldhoen, K. M., Veldhoen, N. J., Carter, N. L., Roth, W.-M. & Hawryshyn, C. W. 2008a Effects of exogenous thyroid hormones on visual pigment composition in coho salmon (*Oncorhynchus kisutch*). *J. Exp. Biol.* **211**, 2134–2143. (doi:10.1242/jeb.009365)
- Temple, S. E., Veldhoen, K. M., Haimberger, T. J., Phelan, J. T., Veldhoen, N. J. & Hawryshyn, C. W. 2008b Ontogenetic changes in cone photoreceptor spectral absorption and opsin expression in coho salmon (*Oncorhynchus*

- kisutch*). *J. Exp. Biol.* **211**, 3879–3888. (doi:10.1242/jeb.020289)
- Timmermans, P. J. A. 2000 Prey catching in the archer fish: marksmanship, and endurance of squirting at an aerial target. *Netherlands J. Zool.* **50**, 411–423.
- Timmermans, P. J. A. 2001 Prey catching in the archer fish: angles and probability of hitting an aerial target. *Behav. Process.* **55**, 93–105. (doi:10.1016/S0376-6357(01)00172-3)
- Timmermans, P. J. A. & Souren, P. M. 2004 Prey catching in archer fish: the role of posture and morphology in aiming behavior. *Physiol. Behav.* **81**, 101–110. (doi:10.1016/j.physbeh.2004.01.010)
- Timmermans, P. J. A. & Vossen, J. M. 2000 Prey catching in the archer fish: does the fish use a learned correction for refraction? *Behav. Process.* **52**, 21–34. (doi:10.1016/S0376-6357(00)00107-8)
- Wagner, H. 1990 *Retinal structure of fishes. The visual system of fish*. London, UK: Chapman & Hall.
- Wood, P., Partridge, J. C. & Grip, W. 1992 Rod visual pigment changes in the elver of the eel *Anguilla anguilla* L. measured by microspectrophotometry. *J. Fish Biol.* **41**, 601–611. (doi:10.1111/j.1095-8649.1992.tb02686.x)
- Yoshizawa, T. 1994 Molecular basis for color vision. *Biophys. Chem.* **50**, 17–24. (doi:10.1016/0301-4622(94)85016-X)