

## RESEARCH ARTICLE

# Comparison of functional and anatomical estimations of visual acuity in two species of coral reef fish

Amira N. Parker<sup>1,\*</sup>, Kerstin A. Fritsches<sup>1</sup>, Cait Newport<sup>1,2</sup>, Guy Wallis<sup>3</sup> and Ulrike E. Siebeck<sup>1</sup>

## ABSTRACT

The high-contrast, complex patterns typical of many reef fish serve several purposes, including providing disruptive camouflage and a basis for vision-based communication. In trying to understand the role of a specific pattern, it is important to first assess the extent to which an observer can resolve the pattern, itself determined, at least in part, by the observer's visual acuity. Here, we studied the visual acuity of two species of reef fish – *Pomacentrus amboinensis* and *Pseudochromis fuscus* – using both anatomical and behavioural estimates. The two species share a common habitat but are members of different trophic levels (predator versus herbivore/omnivore) and perform different visual tasks. On the basis of the anatomical study, we estimated visual acuity to lie between 4.1 and 4.6 cycles deg<sup>-1</sup> for *P. amboinensis* and 3.2 and 3.6 cycles deg<sup>-1</sup> for *P. fuscus*. Behavioural acuity estimates were considerably lower, ranging between 1.29 and 1.36 cycles deg<sup>-1</sup> for *P. amboinensis* and 1.61 and 1.71 cycles deg<sup>-1</sup> for *P. fuscus*. Our results show that two species from the same habitat have only moderately divergent visual capabilities, despite differences in their general life histories. The difference between anatomical and behavioural estimates is an important finding as the majority of our current knowledge on the resolution capabilities of reef fish comes from anatomical measurements. Our findings suggest that anatomical estimates may represent the highest potential acuity of fish but are not indicative of actual performance, and that there is unlikely to be a simple scaling factor to link the two measures across all fish species.

**KEY WORDS:** Operant conditioning, Retinal topography, Visual behaviour, Spatial frequency, Marine signalling, Spatial resolution

## INTRODUCTION

Many coral reef fish use visual signalling for a range of behaviours, including maintaining and defending territories, recognizing individuals and/or species and attracting potential mates (Cott, 1940; Endler, 1991; Marshall and Vorobyev, 2003; Siebeck, 2004; Siebeck et al., 2010). Furthermore, prey species often become aware of the presence of a predator through visual signals. In order to survive, they need to identify the nature of the potential attack and respond appropriately (Kelley and Magurran, 2003). Errors in the interpretation of these signals by the receiver can be costly and lead to a loss of fitness. Because the visual system of fish has evolved in

response to a range of social and environmental pressures (Dobberfuhl et al., 2005), the visual capabilities of different species are highly variable (Lythgoe, 1979; Endler, 1990, 1993), and can be described using a range of measures including (but not limited to) acuity, temporal resolution and absolute visual sensitivity. Visual acuity, or spatial resolution, is a measure of the minimum separable angle that can be resolved by the eye (Neave, 1984) and is one of the most common measures to assess the visual capability of an animal (Reymond, 1985; Harman et al., 1986; Collin and Pettigrew, 1989; Aho, 1997; Haug et al., 2010). Knowledge of visual acuity of an animal allows us to evaluate the level of detail an animal can see in a visual scene, which is important if we wish to understand various aspects of their visual behaviour independently of the human visually guided behaviour perception.

Differences in lifestyle and habitat complexity lead to differences in visual tasks and, with that, high variability in acuity among species. For example, the wedge tailed eagle (*Aquila audax*), has a high behavioural acuity of 143 cycles deg<sup>-1</sup>, as it relies on vision to spot prey from a great distance (Reymond, 1985). In contrast, the domestic chicken (*Gallus gallus domesticus*), which feeds at a much closer distance, has a much lower behavioural acuity of 7 cycles deg<sup>-1</sup> (Jarvis et al., 2009). Acuity is also thought to be influenced by an organism's light environment (Lee and O'Brian, 2011). A study conducted on a temperate (*Hippocampus abdominalis*) and tropical (*Hippocampus taeniopterus*) species of seahorse found that tropical species had a higher acuity compared with their temperate counterparts, despite living in similar habitats. The difference in acuity may be attributable to the two species' light environment, as temperate waters tend to be more turbid with a narrow spectrum of light, whilst tropical coral reefs have a broad spectrum of light (Mosk et al., 2007). The increased visual resolution of *Hippocampus taeniopterus* may enhance its ability to capture prey in the bluer water of the tropical environment (Lee and O'Brian, 2011). Amongst fishes, reports indicate a wide range in spatial resolution capabilities, commensurate with their wide range of habitats and lifestyles (for review, see Douglas and Hawryshyn, 1990). One study, which used anatomical measurements to estimate acuity for 15 species of reef fish from varying habitats, found that acuity varied between 4 and 27 cycles deg<sup>-1</sup> (Collin and Pettigrew, 1989). Although in some instances fish living in similar habitats may have similar acuities (for example, *Halophryne diemensis* and *Pomacanthus semicirculatus*: 4 and 7 cycles deg<sup>-1</sup>, respectively), this is not always the case. Species found inhabiting open water can have much larger differences in acuity (for example, *Lethrinus chrysostomus* and *Gymnocranius bitorquatus*: 19 and 27 cycles deg<sup>-1</sup>, respectively) (Collin and Pettigrew, 1989).

The visual acuity of fish has been measured using a range of behavioural (Brunner, 1934; Yamanouchi, 1956; Nakamura, 1968; Hodos and Yolen, 1976; Hairston et al., 1982; Neave, 1984; Pankhurst et al., 1993; Neumeier, 2003b; Haug et al., 2010; Lee and O'Brian, 2011; Champ, 2012) and anatomical methods (Hodos and

<sup>1</sup>Laboratory for Visual Neuroethology, School of Biomedical Sciences, University of Queensland, St Lucia, QLD 4072, Australia. <sup>2</sup>Department of Zoology, University of Oxford, Oxford OX1 3PS, UK. <sup>3</sup>Centre for Sensorimotor Performance, School of Human Movement and Nutrition Sciences, University of Queensland, St Lucia, QLD 4072, Australia.

\*Author for correspondence (aparker@uq.edu.au)

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Yolen, 1976; Collin and Pettigrew, 1989; Fritsches et al., 2003; Matsuda et al., 2005, 2008; Theiss et al., 2007; Litherland and Collin, 2008; Kino et al., 2009; Carton and Vaughan, 2010; Temple et al., 2010; Lee and O'Brian, 2011; Champ, 2012). However, because of the many differences in methodology, comparisons between studies are difficult. In cases where both anatomical and behavioural methods have been used to measure acuity, there are some examples where the acuity estimates are similar (Brunner, 1934; Marc and Sperling, 1976; Neumeyer, 2003b; Temple et al., 2013). However, it is more common that behavioural acuity estimates are lower than acuity estimated from anatomical measures (see Table 1 for review). There are several possible explanations for this discrepancy. Anatomical measures do not account for higher order processing beyond the retina, which would lead to an underestimate of behavioural abilities. At the same time, this approach also fails to take into account any optical properties of the eye that may limit acuity (Browman et al., 1990; Haug et al., 2010; Lee and O'Brian, 2011), possibly leading to an overestimate of the capabilities of the fish. Behavioural estimates of visual acuity are likely to yield a more accurate estimate of an animal's actual visual ability as behaviour is the output of both the retinal and neural systems (Browman et al., 1990; Douglas and Djamgoz, 1990). However, behaviour experiments can be impractical for species that cannot be tested in captivity. In addition, the results of behavioural experiments can be variable and time consuming because of differences in motivation amongst individuals (Dickinson et al., 1995; Niv et al., 2006). As a result, fewer than 15 studies have compared behavioural and anatomical estimates of acuity (but see Table 1 for a review).

The purpose of the present study was to investigate the visual acuity of two species of reef fish using both anatomical and behavioural techniques. Photoreceptor and ganglion cell densities within the retina were measured to provide an estimate of the maximum optical acuity of the visual system. Behavioural experiments were then used to determine how the acuity of each species is expressed functionally. Fish were trained to discriminate between horizontal and vertical broad-spectrum gratings. The spatial frequency of the gratings was then systematically increased until the fish could no longer discriminate the gratings. The Ambon damselfish (*Pomacentrus amboinensis*; Bleeker 1868) and one of its predators, the yellow dottyback (*Pseudochromis fuscus*; Müller and Troschel 1849), were selected as test species as they often share the same habitat, but come from different trophic levels and undertake different visual tasks. Also, both species of fish adapt well to captivity and readily perform visual discrimination tasks (A.N.P., unpublished results; Siebeck, 2004; Siebeck et al., 2008; Siebeck et al., 2010; Cripps et al., 2011).

*Pomacentrus amboinensis* is known to use unique facial patterns for species and individual recognition (Siebeck et al., 2010). These facial patterns are complex and discrimination requires the ability to discern fine detail. In contrast, *P. fuscus* is a solitary, opportunistic predator known to prey on slow-moving benthic crustaceans as well as newly settled fish, including juvenile *P. amboinensis* (McCormick and Meekan, 2007; Cortesi et al., 2015a). Whilst *P. fuscus* must learn to avoid unpalatable or poisonous prey, they have no obvious patterns themselves that differ between individuals, nor are they able to see the facial patterns on *P. amboinensis* as they are UV blind (Siebeck and Marshall, 2001; Cortesi et al., 2015b). The difference in visual tasks performed by each species suggests that, despite living in the same visual environment, *P. amboinensis* are expected to have a slightly higher acuity, at least for the static patterns forming the focus of this study.

## MATERIALS AND METHODS

### Animal collection and housing

Male fish of each species (7–8 cm standard length) were caught in shallow reefs near Lizard Island, on the Great Barrier Reef (14°40'S 145°28'E) using custom-made hand nets during scuba diving, with permission from the Great Barrier Reef Marine Park Authority (G08/27055.1) and the Queensland Fisheries Service (PRM377271). Fish were sent to Brisbane (in individual plastic bags half-filled with fresh seawater and topped up with oxygen) using a commercial aquarium trader (Cairns Marine Aquarium, Stratford, QLD, Australia). All experiments were conducted in accordance with the University of Queensland Animal Ethics guidelines (AEC approval numbers: VTHRC/194/08/ARC/UQ, VTHRC/212/09/AUSTRALIA PACIFIC FOUNDATION/U). Because of their territorial nature, all fish were housed in separate aquaria (35×26×20 cm), each containing a PVC pipe (10 cm length, 5 cm diameter) that served as a shelter. High water quality was maintained as each aquarium contained an internal water filter (Aqua one 101F), which was cleaned every second day. In addition, regular water changes were carried out every second day. Fish were fed a mixture of water and fish flakes (HBH: flake frenzy marine flakes, Springville, UT, USA) daily as part of the experiments. Also, because of their territorial nature, the fish were not moved to a different aquarium for testing but were instead tested in the aquarium that contained their new territory. The food reward was administered by hand with a syringe and plastic tubing. The aquarium room was illuminated using standard fluorescent 60 W strip lighting (ambient light: 349 lx) on a 12 h:12 h light:dark cycle. Following the behavioural experiments, fish were kept in captivity at the University of Queensland and used for further visual behavioural experiments.

### Retinal anatomy

#### Photoreceptor and ganglion cell density counts

Six light-adapted fish from each species were killed by severance of the spinal cord. The standard length of each fish was recorded to the nearest millimetre. Once enucleated, the diameter of the eye was measured to the nearest 0.1 mm along the dorsal–ventral, axial (corneal–scleral) and equatorial (anterior–posterior) axis (Ullmann et al., 2012). The technique for dissecting the retinal layer from the eye and subsequent mounting was largely derived from Stone (1981) and only variations to the technique are described below. A ventral orientation slit was initially made to ensure correct orientation of the retina. Once dissected, the vitreous humour and retinal pigment epithelium were gradually removed, and the retina was then fixed in 4% paraformaldehyde in 0.1 mol l<sup>-1</sup> phosphate buffer solution (pH 7.4) and stored at 4°C for 1–2 h. A wall surrounding the flattened retina was made with duct tape, and 100% glycerol was then added. A coverslip was placed over the retina and sealed with nail polish.

The same individual retina used for photoreceptor cell topography was also used for ganglion cell topographic studies. Mounting and drying methods followed procedures described by Curcio et al. (1987). After retinas were mounted and dried, they were rehydrated through a descending alcohol series followed by a 2 min rinse in distilled water. Each retina was then Nissl stained using a 0.01% Cresyl Violet solution (pH 4.3) for 15 s. After staining, slides were dipped in 0.025% acetic acid solution to remove any excess Cresyl Violet. Slides were then dehydrated in an ascending alcohol series before being cleared in xylene. Each retina was coverslipped in a DEPEX mounting medium (Sigma-Aldrich, St Louis, MO, USA).

#### Topography maps

Initially, an outline of each retina for each cell layer examined was mapped onto 1.0 mm<sup>2</sup> lined graph paper, viewing the retina with a

**Table 1. Visual acuity estimates of fish species for which both anatomical and behavioural estimates were determined**

Species	Common name	Visual acuity (cycles deg <sup>-1</sup> )		Ecology	Reference
		Behaviour	Anatomy		
Photoreceptor cells					
<i>Danio rerio</i>	Larval zebrafish	0.16 (OR)	0.24 (PRS)	Freshwater Omnivorous Prey sp.	Haug et al., 2010
<i>Toxotes chatareus</i>	Archerfish	3.33 (MDA)	3.57 (PRS)	Freshwater Omnivorous Predatory sp.	Temple et al., 2013
<i>Microcanthus strigatus</i>	Convict fish	13.04 (MDS)	16.67 (PRS)	Marine/non-pelagic Omnivorous Prey sp.	Yamanouchi, 1956
<i>Lepomis macrochirus</i>	Bluegill sunfish	4.3 (RD)	22.2 (PRS)	Freshwater Omnivorous Prey sp.	Hairston et al., 1982
<i>Oncorhynchus mykiss</i>	Rainbow trout	0.625 (OR)	4.35 (PRC)	Marine/freshwater Carnivorous Predatory sp.	Carvalho et al., 2004
<i>Forsterygion varium</i>	Striped triplefin	1.11 (RD)	6.67 (PRC)	Marine/non-pelagic Omnivorous Prey sp.	Pankhurst et al., 1993
<i>Phoxinus laevis</i>	Minnow	5.45 (MDA)	5.45 (PRC)	Freshwater Omnivorous Prey sp.	Brunner, 1934
<i>Carassius auratus</i>	Goldfish	2 (MDA)	2.2 (PRC)	Freshwater Herbivorous Prey sp.	Marc and Sperling, 1976; Neumeyer, 2003a
<i>Katsuwonus pelamis</i>	Skipjack tuna	10.7 (MDA)	26 (PRC)	Marine/pelagic Carnivorous Predatory sp.	Tamura and Wisby, 1963; Nakamura, 1968
Ganglion cells					
<i>Hippocampus abdominalis</i>	Big-belly seahorse	0.06 (RD)	6.12 (GCC)	Marine/non-pelagic Carnivorous Predatory sp.	Lee and O'Brian, 2011
<i>Hippocampus taeniopterus</i>	Common seahorse	0.09 (RD)	6.64 (GCC)	Marine/non-pelagic Carnivorous Predatory sp.	Lee and O'Brian, 2011
<i>Rhinocanthus aculeatus</i>	Picasso triggerfish	1.75 (MDA)	3.4 (GCC)	Marine/non-pelagic Carnivorous Predatory sp.	Champ et al., 2013
<i>Astronotus ocellatus</i>	Oscar	11.32 (MDA)	12 (unknown)	Freshwater Omnivorous Predatory sp.	Weiler, 1966

Anatomical acuity is shown based on photoreceptor cells (top) and ganglion cells (bottom). The method used in each particular study is noted: OR, optomotor/optokinetic response; RD, reactive distance; MDA, minimum distinguishable acuity; PRS, photoreceptor spacing; PRC, photoreceptor counts; GCC, ganglion cell counts.

Zeiss Axioskop compound microscope (40× magnification). For photoreceptor counts, photographs were taken at 100× magnification with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Photographs were taken every 1 and 0.5 mm in low- and high-density areas, respectively, and analysed by counting the double and single cone photoreceptors using a standardized grid. For ganglion cells, counts were made at the same distances at 100× magnification, resulting in approximately 150–200 points being sampled across the retina. All clearly identifiable neural elements lying within the ganglion cell layer were counted irrespective of size (Collin and Pettigrew, 1988a,b; Litherland and Collin, 2008; Temple et al., 2010; Lee and O'Brian, 2011; Champ et al., 2013). Because of their distinct elongated shape and dense staining (Collin and Pettigrew, 1988c), glial cells were easily identifiable and were excluded from cell counts. Sampling areas at each point were defined by a 10×10 square eyepiece graticule (magnification calibrated for objective used). Cells that touched the top of the left-hand side of the sampling grid were omitted from counts to prevent oversampling, as they would be counted in the previous grid above and to the side.

Several adjustments were made to ganglion cell counts, including accounting for shrinkage and for the inclusion of non-neuronal elements (displaced amacrine cells) (Stone, 1981; Curcio et al., 1987; Collin and Collin, 1988; Mednick and Springer, 1988; Hart, 2002; Fritsches et al., 2003). Final counts were then converted to cells per square millimetre. From the data points for both cell densities, topography maps were constructed linking areas of similar cell density (Stone, 1981). In both photoreceptor and ganglion cell estimates, a density map was then created by linking the number of cells counted at each point to the relevant location on the graphed retinal outline.

#### Anatomical estimates of visual acuity

Summation ratios were calculated between the density of photoreceptors and that of ganglion cells at overlapping points of each cell layer. With the aid of Matthiessen's ratio (Matthiessen, 1880), the anatomical visual acuity, sometimes referred to as spatial resolving power, can be calculated. Assuming a square mosaic, visual acuity was calculated in the regions of highest cell density for



both photoreceptor and ganglion cell layers (for equations, see Collin and Pettigrew, 1989). For the anatomical visual acuity estimate, it was assumed that all of the photoreceptors (double and single cones) in the mosaic were being used for the animal's visual acuity (Matsuda et al., 2008; Haug et al., 2010; Temple et al., 2010, 2013; Champ et al., 2013).

## Behavioural acuity

### Apparatus and stimuli

Experiments were run in the home tank of each fish. A transparent Perspex barrier was placed 8 cm from the stimuli to control the minimum distance at which the fish could examine the stimuli during experimentation. An opaque, white Perspex separator was attached to the middle of the barrier to ensure that the fish could only see one stimulus at a time once it approached the barrier (Fig. 1A). The barrier was placed in the tank before each session and removed upon completion of the session.

The stimuli consisted of a series of square-wave gratings. Gratings were chosen because they have the same brightness and are commonly used to estimate acuity (Yamanouchi, 1956; Nakamura, 1968; Srinivasan and Lehrer, 1988; Macuda et al., 2001; Champ et al., 2013). Widths of gratings (consisting of one black and one white bar) were 10 mm (corresponding to  $0.17 \text{ cycles deg}^{-1}$ ) during training and ranged from 3.33 and 0.87 mm (corresponding to  $0.50$  and  $1.90 \text{ cycles deg}^{-1}$ , respectively) during testing. Stimuli comprised black and white bars of equal size and number printed on paper and laminated ( $2 \times 2 \text{ cm}$ ). When displayed, gratings were always rotated to ensure the black bar was not always at the bottom or to the left. This was to ensure fish did not always select the pattern that had a lower dark centre of gravity.

### Procedure

#### Training

A number of learning steps, following the protocol described in Siebeck et al. (2009), were used to train fish to associate a particular stimulus orientation with a food reward. Briefly, fish were trained to initially feed from a feeding tube attached to a plastic syringe (containing a mixture of fish flakes and water). The next step was to associate the food reward with a particular stimulus (henceforth referred to as the reward stimulus). Fish were only fed when they interacted by tapping or rubbing their mouth against the reward stimulus. Half the fish ( $n=3$ ) were trained to select the horizontal grating and the other half ( $n=3$ ) were trained to the vertical grating. Once fish could successfully associate a food reward with the reward stimulus (by tapping at least two times), and showed their typical anticipatory behaviour such that the food reward could be delivered at the far end of the tank, a distance barrier was added along with the distracter stimulus. Fish were then required to continue to select the

reward stimulus by tapping or rubbing their mouth on the Perspex distance barrier (set 8 cm away from the stimuli) directly in front of the selected stimulus (Fig. 1B). A trial ended when the fish selected the correct stimulus twice in succession. The number of correct and incorrect taps was tallied and used to calculate the frequency of correct choices for each session. Fish were considered to have learned the task when they reached 75% correct choices or greater for at least five consecutive sessions.

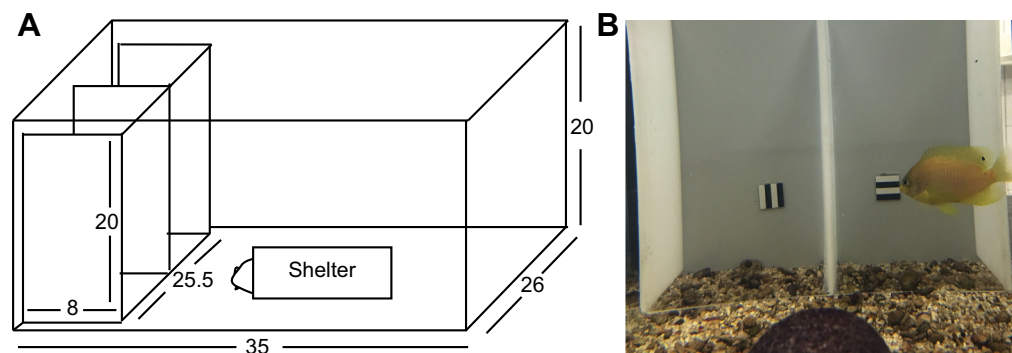
#### Testing

Once each fish within both groups fulfilled the criterion for learning, they advanced to testing. A two-alternative forced choice procedure was used to test the fish, following methods described by Siebeck et al. (2010). The reward and distracter stimuli were shown an equal number of times on either side, with a constraint that the same stimulus was never presented in the same position more than twice in succession. This was done to prevent development of a side bias. Each session began with placement of the stimulus holder and barrier at the far end of the aquarium, with an opaque board positioned in front of the holder to hide the stimuli. Once the fish had moved back to its home, the opaque board was removed and the trial started. This was done to ensure the fish had the same viewing distance at the beginning of each trial. The number of taps the fish made for each stimulus was recorded. A trial ended when the fish selected the correct stimulus twice in succession. After a correct choice, the fish was rewarded using the same method that was used during the training phase. If the fish took longer than 2 min to make a decision, the opaque board was placed in the tank and once fish moved to their home, the trial was repeated. If there was still no response from the fish after 2 min, the session was terminated for that individual. The experimenter would return to this individual to complete the session only after all other fish had completed a full session.

The gratings consisted of vertical and horizontal black and white striped patterns with spatial frequencies ranging between  $0.50$  and  $1.90 \text{ cycles deg}^{-1}$ . The patterns were presented in frequency-matched pairs during five sessions. Each session consisted of eight trials resulting in 40 choices per spatial frequency. Testing began with the lowest spatial frequency ( $0.50 \text{ cycles deg}^{-1}$ ) and then proceeded to successively higher spatial frequencies until the fish could no longer discriminate between the two orientations. The threshold criterion was set at 72.5% correct choices. Upon completion of the experiment, fish were given a further five sessions with the lowest spatial frequency to ensure the decrease in performance was not due to a lack of motivation.

### Calculation of behavioural visual acuity

The spatial frequency (SF) of each pattern was calculated based on the grating size and viewing distance using the following formula,



**Fig. 1. Experimental apparatus.** (A) Schematic diagram of the aquarium, showing the Perspex barrier. Dimensions are given in centimetres. (B) View from the perspective of the experimenter, showing the Perspex barrier, stimuli and a fish performing a trial.

adapted from Nakamura (1968):

$$SF = \frac{1}{\left(2 \tan^{-1} \left( \frac{0.5CW}{D} \right) \right) \left( \frac{180}{\pi} \right)}, \quad (1)$$

where CW is the width of one cycle (in mm) and  $D$  is the minimum viewing distance (96 mm). One cycle is defined as the combined width of one black and one white band. The distance of discrimination in these experiments included the distance from the decision point to the placement of the stimuli, set at 80 mm (Fig. 1B), and the anterior nodal point (the distance from the end of the snout to the anterior surface of the cornea: 16 mm).

### Statistical analysis

The number of correct and incorrect taps was tallied and used to calculate the percentage of correct choices for each spatial frequency tested. Data for each fish were fitted with a logistic function using the Palamedes toolbox (version 0.8.1; <http://www.palamedestoolbox.org>) for Matlab (Mathworks version 2014b). Slope and threshold parameters were allowed to freely vary. The guess rate was fixed at 50% and the lapse rate set such that the function asymptote matched the maximum performance level of each individual fish across all frequencies. From these functions, the threshold of discrimination was then calculated for each fish based on achieving a level of accuracy of 72.5% (chosen as the threshold for significant deviation from chance in a two-alternative forced choice test, based on a binomial test,  $n=40$ ,  $P<0.01$ ).

## RESULTS

### Anatomical estimates of visual acuity

The spatial distributions of photoreceptor and ganglion cells were measured for both *P. amboinensis* and *P. fuscus*. Standard body lengths were on average larger in *P. amboinensis* ( $9 \pm 0.36$  cm, mean  $\pm$  s.d.) than in *P. fuscus* ( $7.23 \pm 0.33$  cm). The mean size of the eye and lens was larger in *P. amboinensis* (eye diameter:  $6.65 \pm 1.9$  mm, lens:  $2.32 \pm 0.1$  mm) than in *P. fuscus* (eye diameter:

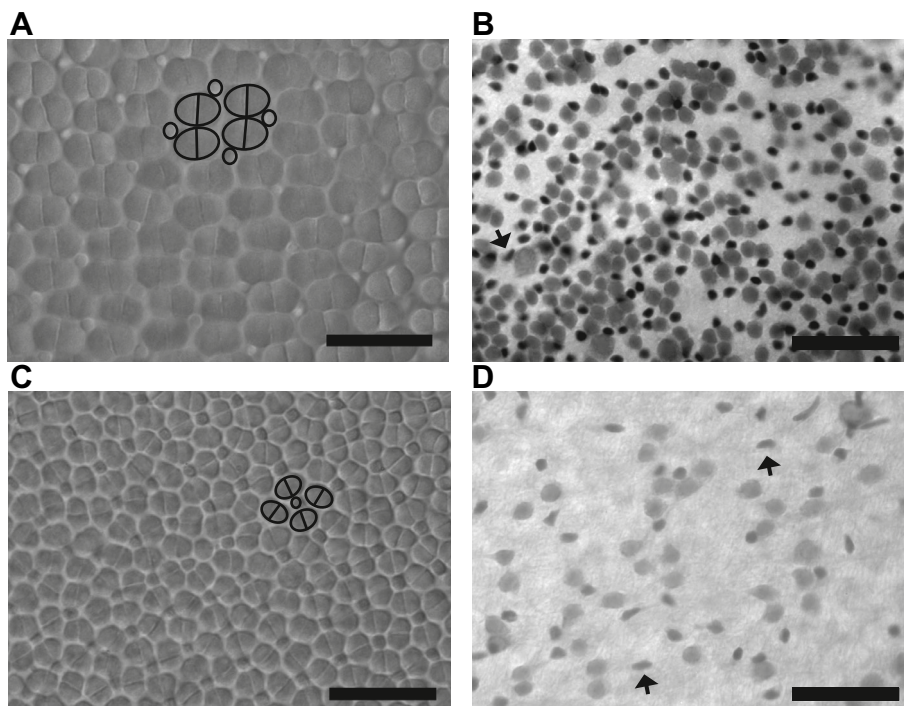
$4.62 \pm 0.22$  mm, lens:  $1.6 \pm 0.15$  mm). Within the photoreceptor layer, cone arrangements in both species generally followed a square mosaic pattern (Fig. 2C), with a single central cone surrounded by four double cones, and at times accessory single cones in the corners of the configuration. Row mosaics were also found in areas of lower cell density (Fig. 2A). Two distinct size groups of ganglion cells were found within the retina of both species (Fig. 2B,D), but were combined into a single density count.

The representative isodensity topography maps of both total photoreceptor cell populations match well with ganglion cell populations within both species, with arrangement of the recorded centro-peripheral cell density gradients varying topographically and quantitatively between *P. amboinensis* (Fig. 3A,B) and *P. fuscus* (Fig. 3C,D). *Pomacentrus amboinensis* had a peak density of cones ( $28,850 \pm 2511$  cells  $\text{mm}^{-2}$ ) and ganglion cells ( $23,100 \pm 2617$  cells  $\text{mm}^{-2}$ ) in the dorso-temporal region of the retina that extended into the centro-temporal region of the retina. A smaller, secondary peak of cell density was also found in the nasal retinal quadrant. In contrast to *P. amboinensis*, *P. fuscus* featured a slight horizontal streak across the dorsal meridian, containing two area centralis with the highest density of cones ( $33,750 \pm 3572$  cells  $\text{mm}^{-2}$ ) and ganglion cells ( $26,550 \pm 4791$  cells  $\text{mm}^{-2}$ ) found in the dorso-temporal quadrant.

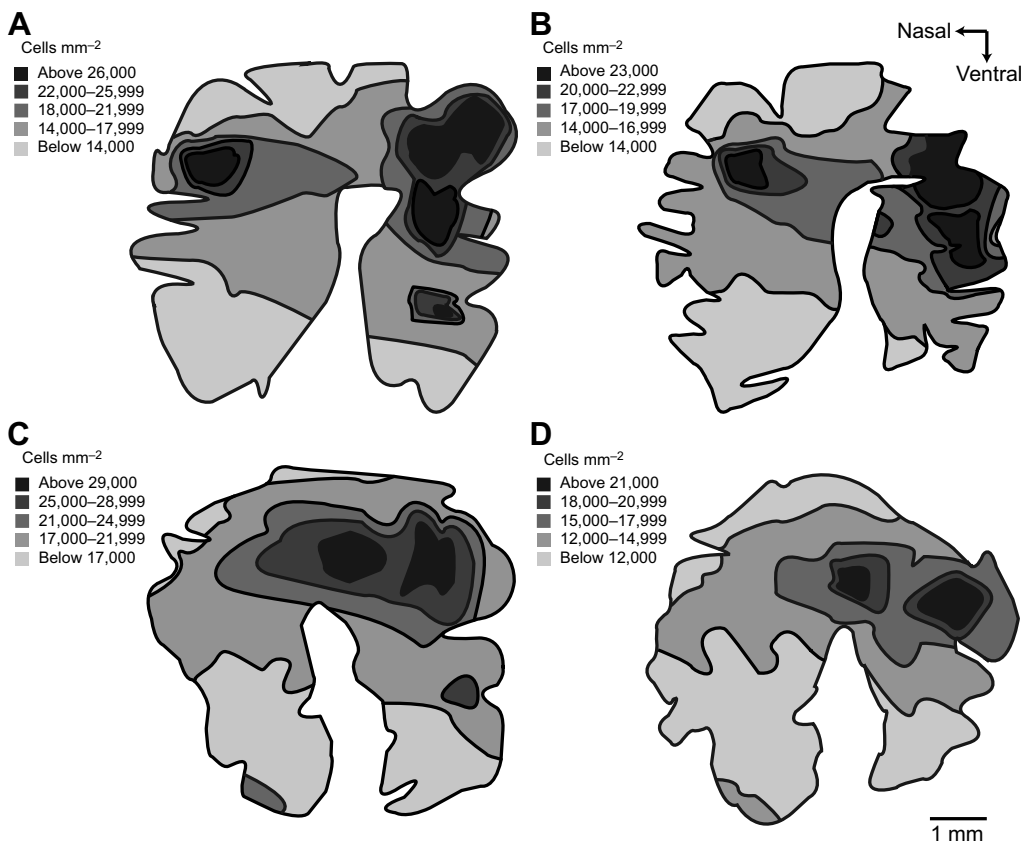
Based on our measurements, the visual acuity calculated from cone photoreceptor counts was generally higher than that estimated from ganglion cell densities. The visual acuity estimates based on cone photoreceptor counts were  $4.6 \pm 0.35$  and  $3.6 \pm 0.27$  cycles  $\text{deg}^{-1}$  for *P. amboinensis* and *P. fuscus*, respectively. The visual acuity estimate based on ganglion cell density was  $4.1 \pm 0.36$  cycles  $\text{deg}^{-1}$  for *P. amboinensis* and  $3.2 \pm 0.32$  cycles  $\text{deg}^{-1}$  for *P. fuscus*. Summation ratios between photoreceptors and ganglion cells varied from 1.4:1 in the areae to more than 2.9:1 in the peripheral retina in *P. amboinensis*, and 1.6:1 in the areae to more than 3.5:1 in *P. fuscus*.

### Behavioural limit of visual resolution

For each species, the percentage of correct choices (based on the tapping distribution) for all fish was grouped and is shown for each



**Fig. 2. Photographs of the photoreceptor and ganglion of each species.** (A,B) *Pomacentrus amboinensis* retina, showing an area with row photoreceptor mosaic (A) and high-density ganglion cells (B). (C,D) *Pseudochromis fuscus* retina, showing an area with square photoreceptor mosaic (C) and low-density ganglion cells (D). Outlines have been included to highlight the position of the double cones (ellipses with central bisectors) and single cones (circles). Arrows point to elongated, cigar-shaped cells presumed to be glial cells, which were not counted in the study. Scale bars, 25  $\mu\text{m}$ .



**Fig. 3. Representative topographic retinal maps of each species.** (A,B) *Pomacentrus amboinensis* photoreceptors (A) and ganglion cells (B). (C,D) *Pseudochromis fuscus* photoreceptors (C) and ganglion cells (D).

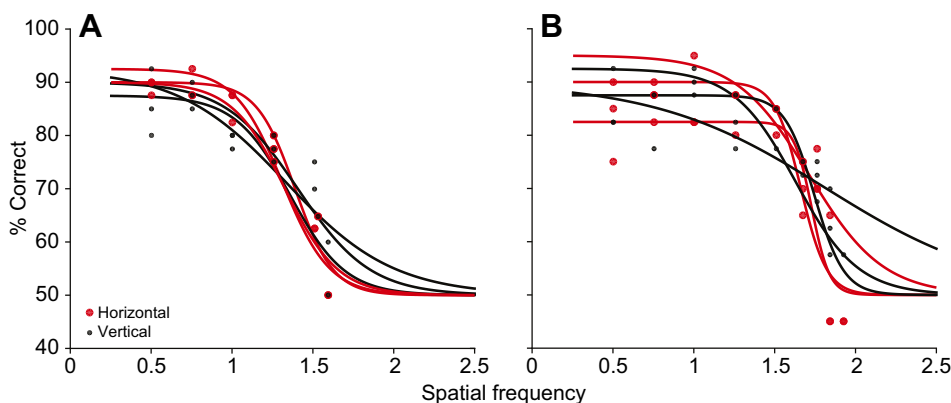
spatial frequency in Fig. 4. For *P. amboinensis*, all individuals ( $n=6$ ) performed best at the lowest spatial frequency (0.50 cycles deg<sup>-1</sup>), reaching on average 81% (correct choice frequencies for the group ranged between 80% and 93%). The group maintained this level of accuracy up to 1.25 cycles deg<sup>-1</sup>, at which point discrimination rate decreased to  $78 \pm 2.23\%$ . Acuity thresholds for individual *P. amboinensis* ranged between 1.29 and 1.36 cycles deg<sup>-1</sup> (72.5% correct choices, binomial test,  $n=40$ ,  $P<0.01$ ). A similar pattern was found for *P. fuscus* individuals ( $n=6$ ). The highest accuracy was observed at the lowest spatial frequency tested (0.50 cycles deg<sup>-1</sup>), reaching between 75% and 93% correct. Performance was maintained for the first five spatial frequencies tested, then dropped rapidly. Acuity thresholds of individual *P. fuscus* ranged between 1.61 and 1.71 cycles deg<sup>-1</sup> (72.5% correct choices, binomial test,  $n=40$ ,  $P<0.01$ ).

There was no significant difference in the acuity threshold for the two groups (trained to the horizontal or vertical grating)

for either species of fish (Wilcoxon rank sum=11,  $n_1=n_2=3$ , n.s. two-tailed, in both cases). Overall, the median threshold for *P. fuscus* was 1.69 cycles deg<sup>-1</sup> and for *P. amboinensis* was 1.29 cycles deg<sup>-1</sup>, with the distributions for the two species being significantly different (Wilcoxon rank sum=21,  $n_1=n_2=6$ ,  $P=0.0022$ , two-tailed).

## DISCUSSION

The aim of this study was to assess the validity of anatomical approaches to estimating visual acuity and to compare measures across species. Our approach involved a combination of classical anatomical and behavioural techniques to estimate the visual acuity of two coral reef species that live in the same visual environment but have different visual behaviours. To facilitate comparisons with other studies, we employed both photoreceptor and ganglion cell counts to calculate the anatomical limit of acuity. In practice, the two anatomical measures produced similar results, with the ganglion cell



**Fig. 4. Results for behavioural acuity experiment.** Fish (A, *P. amboinensis*; B, *P. fuscus*) were trained to horizontal (red) or vertical (black) gratings. Individual dots represent the percentage correct choices of each fish at each of the tested spatial frequencies ( $n=40$ ). Lines show fitted psychometric functions.



measure providing slightly more conservative estimates. As the difference between these two retinal layers reflects the convergence of visual information between them, we focus on the acuity calculated from the ganglion cells in all subsequent comparisons with the behavioural measures.

*Pomacentrus amboinensis* was found to have a behavioural acuity of  $1.29 \text{ cycles deg}^{-1}$ , which was significantly lower than the anatomical limit of  $4.1 \text{ cycles deg}^{-1}$ . *Pseudochromis fuscus* had a slightly higher behavioural acuity of  $1.69 \text{ cycles deg}^{-1}$  but a lower anatomical acuity of  $3.6 \text{ cycles deg}^{-1}$ . The acuity and retinal topography assessed in both species fits within the typical range of shallow water reef fish (Collin and Pettigrew, 1989; Champ et al., 2013). Our results indicate that, despite the fact that the social system of *P. amboinensis* requires that they discern the fine detail of facial patterns of conspecific individuals, their visual abilities are similar to those of *P. fuscus*. Based on their behavioural acuity, *P. amboinensis* can probably resolve the larger components of their facial patterns (approximately 3 mm in diameter) at distances of up to 44 cm away (for calculation of methods, see Marshall, 2000). The smaller facial pattern components around the eye ( $\leq 1 \text{ mm}$  diameter) can be resolved at distances of less than 15 cm. The predator, *P. fuscus*, in contrast, has the potential to resolve pattern components of this size up to 62 and 21 cm, respectively.

It is important to note that our observed acuity measurements are based on data collected from broad-spectrum patterns viewed in the well-lit, clear waters of our aquarium system. To understand the full visual capability of a species, including its ability to discern natural patterns and objects, other visual elements must be taken into account. For example, how a species' visual system processes the contrast between the pattern component/object and its background can affect how a pattern/object is perceived. In addition, contrast depends on a range of variables, including pattern wavelengths and water quality, factors that need to be carefully evaluated when considering specific abilities performed in the context of their habitat (Lythgoe, 1979; Douglas and Djamgoz, 1990).

Looking beyond this study, one should be a little wary when attempting to compare the behavioural results of different studies because visual estimates can depend on the specific method used. For the 13 fish species for which both anatomical and behavioural acuity has been measured, anatomical estimates of acuity were higher than behavioural acuity in seven cases and similar in four cases (see Table 1). The discrepancy could be due to the dependency of the behavioural results on the particular paradigm and stimuli used to determine functional acuity. Srinivasan and Lehrer (1988) found different outcomes in the acuity of honeybees depending on whether radial (sectored) or linear (square-wave) gratings were used. Srinivasan and Lehrer (1988) suggested that the higher acuity found with square-wave gratings is probably due to motion cues as freely flying bees may experience greater stimulation of horizontal motion-sensitive cells when approaching vertical gratings, and vice versa. Triggerfish have also been noted to perform poorly in acuity tests when trained to circular stimuli as opposed to grating stimuli (Champ et al., 2013). The authors suggested that this might be due to the fact that circular stimuli used in the experiment subtended a smaller angle on the retina than the much larger grating stimuli. Overall, it appears that there is no perfect way to measure functional acuity, as a number of factors, such as the experimental paradigm as well as the stimuli used, can influence acuity measures. For example, the ability to detect the misalignment of two lines yields a measure of hyperacuity rather than acuity. In humans, this task can be solved by single ganglion cells that are potentially much bigger than the stimulus (Westheimer, 1976; Fahle, 2002). Also,

studies in humans suggest that the acuity measured using the Landolt C test is around twice as high as that established using more conventional grating stimuli. This can be explained by the fact that the C-shaped test patterns also possess energy in frequencies lower than the gap in the test shape (Bondarko and Danilova, 1997). This, in turn, might explain why one study reported similar results for anatomical and behavioural measures ( $3.23\text{--}3.52 \text{ cycles deg}^{-1}$ ) (Temple et al., 2013). The Landolt C test may not be a valid measure of acuity as a different mechanism may be at work (i.e. hyperacuity). Overall, it is possible that the behavioural acuity measured in our study could be improved to match anatomical acuity if a different type of behavioural test or different stimulus set was used. For example, repeating the experiment using a grating that is tested against a uniform grey stimulus may reduce the discrepancy between behavioural and anatomical results (Reymond, 1985, 1987; Neumeyer, 2003b). Using this method, Neumeyer (2003b) found similar results when measuring acuity in the goldfish. However, it is possible that there were slight differences in brightness between the stimuli and that the goldfish in fact were basing their decision on brightness discrimination at spatial frequencies beyond their acuity.

Alternatively, it is possible that the anatomical values in our case in fact overestimate acuity if the assumption of the validity of Matthiessen's ratio is incorrect for the study species (Matthiessen, 1880). Matthiessen's ratio of 2.55 is commonly used to calculate anatomical visual acuity and was also used in this study. Matthiessen found that although focal lengths varied between 2.2 and 2.8 lens radii, nearly all fish have focal lengths of about 2.5 lens radii (Walls, 1943). However, even if ratios were reduced to 2.2 in both species studied here, acuity is only reduced by  $\sim 0.4 \text{ cycles deg}^{-1}$ . Furthermore, for anatomical acuity to match the acuity estimated from behavioural experiments, focal length would need to be reduced to  $<0.5$ , which is unlikely. One thing that is certainly true is that the anatomical methods fail to take into account any processing that occurs beyond the retina. In the case of hyperacuity, this later processing stage is crucial to determining behavioural ability.

The analysis of retinal topography in the two species indicates similar areas of visual importance, which is not surprising as it fits with the 'terrain theory' described by Hughes (1977). According to this theory, the topography of cells across the retina is a representation of the symmetry of the habitat (Hughes, 1977). Species living in complex environments, such as coral reefs, often possess peak densities of retinal ganglion cells, known as area centralis, in the temporal or dorso-nasal visual field (Collin and Pettigrew, 1988a). In contrast, pelagic species or ones that live over sandy bottoms near coral reefs have an uninterrupted view of the sand–water horizon and tend to possess a horizontal streak of high cell density (Collin and Pettigrew, 1988b). Both species investigated here fit the general pattern found for individuals co-existing in the same highly complex visual environment, such as the reef (Collin and Pettigrew, 1988a; Lee and O'Brian, 2011; Champ, 2012).

The highest area of cell density recorded for *P. amboinensis* was found in the dorso-temporal region (extending centrally), with a slightly smaller area found in the dorso-nasal region. The location of high cell density in *P. amboinensis* is consistent with Ali and Anctil's (1976) findings on other species from the same family (Pomacentridae). For *P. amboinensis*, the location of these areas may facilitate the detection of small objects, such as algae on the substrate and/or zooplankton within the water column. Furthermore, the extension of this area centralis to the central field may enhance individual and species recognition of fish, which can be best identified using their patterns (Siebeck, 2004; Siebeck et al., 2010).

In addition, the dorso-nasal region with slightly lower cell density projects to the caudal visual space. Collin and Pettigrew (1988a) suggest that this area centralis, found also in the staghorn damselfish, *Amblyglyphidodon curacao*, may enable fish to negotiate the finger-like projections of the staghorn coral in escape situations. This zone may also be important for predator detection and/or territorial defence. As *P. amboinensis* live in small groups with a single dominant male surrounded by a number of females, this area centralis may help males to keep an eye on their females and eggs from potential predators (McCormick, 1999).

*Pseudochromis fuscus* was found to possess a high density of cells across the dorsal meridian and two small areas of high cell density in the dorsal–temporal region of the visual field. The band of high cell density across the meridian indicated the presence of a weak horizontal streak with a prominent area of high cell density in the same location as in *P. amboinensis*. This increase of cell density across the retinal meridian may allow *P. fuscus*, a sit and wait predator, to maintain their position and spend their time scanning their environment for benthic prey and potential threats. The area of high cell density found in the dorsal–temporal region may enhance prey capture once prey is detected, which has also been described in *Balistoides conspicillum* and *Aulostoma chinensis* (Collin and Pettigrew, 1988b).

It is assumed that predatory fish have higher acuities than prey fish (Collin and Pettigrew, 1989). Our results show comparable values for the two species, although note that the behavioural measures, at least, were statistically significantly different and not in the direction predicted by Collin and Pettigrew (1989) (1.29 and 4.1 cycles deg<sup>−1</sup> recorded for *P. amboinensis*; 1.69 and 3.6 cycles deg<sup>−1</sup> recorded for *P. fuscus*, using anatomical and behavioural measures, respectively). In practice, the differences are minor when the size of the fish (in particular, lens size) is taken into account. A number of studies record visual acuities that vary in proportion to eye size and lens diameter (Walls, 1943; Collin and Pettigrew, 1989; Kiltie, 2000; Bozzanao et al., 2001). Having a larger lens provides fish with the ability to detect smaller prey or the same size prey at a greater distance (Bozzanao et al., 2001). In this study, the lens size in *P. amboinensis* was on average slightly larger than that of *P. fuscus*, resulting in the higher anatomical acuity. If *P. fuscus* possessed the same lens size as *P. amboinensis*, acuity would only vary between the two species by ~0.2 cycles deg<sup>−1</sup>, which is a negligible difference.

When considering the different values of acuity, one should bear in mind that the acuities were calculated based on the controlled minimum distance. Although this was fixed throughout experiments, it is not known at what distance the fish made the decision about which stimulus to choose. Whilst it could be argued that fish may have made their decision from their home, this is unlikely in this case. Generally speaking, during each trial, fish would often move towards the Perspex barrier looking at both stimuli before making a choice. Furthermore, if acuity is recalculated taking into account the added distance the home may give (i.e. 5 cm), it would only increase from 1.35 to 2.05 cycles deg<sup>−1</sup> in *P. amboinensis* and from 1.71 to 2.6 cycles deg<sup>−1</sup> in *P. fuscus* (values based on highest acuity recorded in each species). Whilst this does increase the acuity value, a discrepancy is still found between anatomical and behavioural results.

The behavioural visual acuity estimates for *P. amboinensis* and *P. fuscus* are similar to those found in the goldfish (Hester, 1968; Wilkinson, 1972; Neumeyer, 2003b) and triggerfish (Champ et al., 2013). These studies reported acuities between 1.5 and 2.0 cycles deg<sup>−1</sup>. Interestingly, these values are based on different

arbitrary thresholds for calculating behavioural acuity. In fact, in addition to the previously described sources for variability in the reported behavioural acuity measures, some variability in the literature is also likely to stem from the definition of the threshold. Detection thresholds are most commonly set at 70% and 75% discrimination accuracy (Hodos and Yolen, 1976; Neumeyer, 2003b; Temple et al., 2013). However, detection thresholds as high as 80% (Weiler, 1966) and as low as 65% accuracy (Champ et al., 2013) have also been reported. It is possible that pre-determined thresholds may contribute to the mismatch between values for anatomical and behavioural acuity. If we applied the threshold criterion of 65% used by Champ et al. (2013) to our study, behavioural acuity would be increased to 1.44 and 1.76 cycles deg<sup>−1</sup> from 1.29 and 1.69 cycles deg<sup>−1</sup> for *P. amboinensis* and *P. fuscus*, respectively. We followed recently published studies in which a threshold of 72.5% was used (Lind and Kelber, 2011; Potier et al., 2016). It is important to remember that by increasing the number of sessions, any accuracy level different from chance can become statistically significant, and that therefore the threshold also depends on the number of trials and sessions carried out. We used 40 trials per spatial frequency, which was identical to Lind and Kelber (2011) but higher than Potier et al. (2016).

In summary, this study is one of three that we know of that explores visual acuity for more than one species of reef fish using both behavioural and anatomical methods. We have highlighted some of the problems associated with the different methods used in the field and believe that there is a need to standardize methods to facilitate comparison and knowledge transfer across species. It is likely that the large differences observed between behavioural and anatomical visual acuity are not only a reflection of the optical properties (i.e. lens size, focal length) of individuals or species but also in fact indicative of mechanisms that occur post-retina; however, this requires further experimentation. Our data also suggest that environmental complexity may be a more important driver for visual acuity than life history, as the predator and prey species studied here were found to have similar visual capabilities.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.N.P., K.A.F., U.E.S.; Methodology: A.N.P., K.A.F., U.E.S.; Formal analysis: A.N.P., G.W., U.E.S.; Investigation: A.N.P.; Resources: K.A.F., U.E.S.; Writing - original draft: A.N.P.; Writing - review & editing: A.N.P., K.A.F., C.N., G.W., U.E.S.; Visualization: A.N.P., U.E.S.; Supervision: K.A.F., U.E.S.; Project administration: U.E.S.; Funding acquisition: K.A.F., U.E.S.

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