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# ANOMALOUS PUPILLARY RESPONSES TO M-CONE ONSETS ARE LINKED TO L:M RATIO

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## ABSTRACT

M-cone stimulation induces a pupil constriction to stimulus offset whereas, with L-cones, the pupil responds conventionally, with a constriction to onset. To test the possibility that this paradox is linked to L:M ratio we measured the strength of the effect by injecting a variable amount of positive or negative luminance contamination either side of M-cone isolation and identifying a balance point at which the pupil responded equally to onset and offset. Nineteen individuals were recruited. In observers with low L:M ratio, the paradoxical effect was weak. There was a significant relationship ( $r^2 = 0.561$ ) between the balance point and L:M ratio. The effect is likely to be linked to strong inhibitory signals associated with cone opponent pathways.

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## 1. INTRODUCTION

A step-wise increase in isolated long wavelength (L) cone excitation generates a conventional pupillary constriction. However, when the medium wavelength (M) cones are modulated in isolation, the main pupillary constriction is to the offset, with minimal response to the onset [1, 2]. This supports our previous studies, in which we showed that this paradoxical behavior has an electrophysiological corollary, in that an offset in M-cone activation generates a characteristic onset ERG response [3, 4], and a psychophysical corollary, since an increment in isolated M-cone luminance is perceived as a decrement in brightness [5]. We have previously speculated about the origin of this phenomenon. Control studies have shown that it is not affected by the background adapting chromaticity so seems to be independent of dominant wavelength [5]. What remaining property of L and M cells might be at work? Other than their spectral tuning, there is no reason to suppose their response properties differ, so it does not seem to be intrinsic to the photoreceptors. One important difference, seen in most human retinæ, is the predominance of L-cones, which we will discuss later. In this study we set out to test the hypothesis that the strength of the anomalous pupillary response seen when the M-cones are decoupled from L-cones might be influenced by the relative numbers of L- and M-cones in an individual retina.

There is a large body of evidence suggesting that the basis of red-green discrimination lies in the post-receptoral comparison of the outputs of L- and M-cones. Midget bipolar cells and midget ganglion cells provide the neural substrate for this process [6-8]. The conventional view is that the cone-opponent pathway receives inputs of opposite sign from L- and M-cones. Thus the retinal circuitry that carries colour information is spectrally opponent, so that signals generated by some wavelengths are excitatory and by others are inhibitory. Simple linear models have been developed which take the form

$$(r-g)_\lambda = aL_\lambda - bM_\lambda$$

where (r-g) is the output of a red-green cone opponent channel,  $a$  and  $b$  are appropriately chosen coefficients and  $L$  and  $M$  are cone fundamentals. This type of analysis has been shown to account for many of the classical threshold-based psychophysical observations, for example by Ingling [9]. A version of these equations also accounts for the expected colour shifts seen in the peripheral retina as described by Murray et al [10], using asymmetrical colour matching data. This technique has shown that cone opponent activity is clearly present at eccentricities of between 18 and 20° [11, 12]. Using a different psychophysical method based on contrast sensitivity, Mullen and Kingdom [13] reported  $L$  and  $M$ -cone activity within 18° of the fovea. These papers show how the observations match those of the underlying physiology [e.g. 14]. Note that Shevell and Martin [15] have produced a highly readable contemporary commentary comparing opponent models based on psychophysical data with those founded in physiology.

Although the total numbers of cones is similar between individuals [16], the relative number of  $L$  and  $M$  cones varies markedly. Direct evidence for this comes from adaptive optics-based measurements [17], but there is substantial indirect evidence. By combining electroretinogram data with psychophysics and retinal densitometry, Kremers et al [18] reported wide variations in  $L:M$  ratio. Hagstrom [19] analysed the  $L:M$  cone pigment ratio using mRNA from donor eyes and showed substantial individual differences in both central and peripheral retina. Brainard et al. [20] describe a range of  $L:M$  ratios from 0.82 to 9.71 in colour-normal males and Carroll et al. [21] combined individualized ERGs with analysis of the opsin gene array to show that although the mean  $L:M$  ratio is around 2, 80% of their 62 colour-normal males had a ratio between 1 and 4 with similar distributions and ranges to other studies.

The issue of  $L:M$  ratio in humans has been investigated for many years and, for the most part, the functional consequences remain elusive. Why, for example, do those with 10 times more  $L$ -cones than  $M$  cones not see the world with a reddish hue? The answer is partly that, as revealed by the modelling, the cone opponent signal is based on the difference between  $L$ -cone and  $M$ -cone output signals and, as is well known, there is substantial overlap in their action spectra. Furthermore it has been shown that the effects of the markedly different  $L:M$  ratios are offset by a cortex-based normalisation process as described by Neitz et al. [22]. Our ERG studies confirm that the manifestation of the numerical  $L:M$  imbalance occurs prior to this normalization and must occur at an early stage of the retino-cortical pathway. If we make the pragmatic assumption that the pupillometric and psychophysical observations of the  $M$ -cone paradox are based on the same mechanism as the ERG findings, it follows that the pupil is blind to the normalization process. More recently, Welbourne et al. [23] found a seasonal compensation in unique yellow in 67 observers and this almost certainly utilizes the same long term adaptability mechanism. Furthermore, unique green is influenced by iris colour, again suggesting the existence of a compensatory mechanism based on the spectral mix of retinal illumination [24].

The Pupillary Light Reflex (PLR) is recorded when there is an increase or decrease in retinal illumination. Conventionally, the pupil responds with a constriction to an increase in light energy and a dilatation to a decrease. The  $M$ -cone paradox described above is particularly evident when  $M$  cones are isolated from the other cone types using the method of silent substitution, first described in 1972 by Estevez and Spekrijse [25]. Murray et al. [1] measured the strength of the inhibitory  $M$ -cone based activity driving the pupil by determining the amount of luminance bias needed to produce a balance between constricting and dilating pupillary responses. Pupil constrictions to stimulus offset have been described previously using chromatic flashes on a white background [26]. Barbur et al. [27] reported a substantial constriction to the offset of a green on red chromatic opponent stimulus.

Recent work has revealed the role of intrinsically photosensitive retinal ganglion cells (ipRGCs) in controlling the pupil [28]. Paradoxical pupillary responses have been described in the context of ipRGCs [2]. In this study, Woelders et al. described isolated  $S$ - and  $M$ -cone

activation that produced inhibitory input to the pupillary control system whereas L-cones and the melanopic ipRGC response generated an excitatory, conventional pupil contraction. We have previously argued that it is unlikely that ipRGCs contribute to the inhibitory responses they described because their stimulus contained extremely low melanopsin contrast and because their stimuli were of too short duration to stimulate ipRGCs [1].

If, as is suggested above, it is the inhibitory M-cone activity that produces the paradoxical PLR, perhaps related to the relative numbers of L and M cones in the retina, we can predict that the paradoxical response will be minimised (or possibly be absent) in subjects with equal numbers of L- and M-cones. Furthermore, we might expect a direct relationship between the strength of the paradoxical effect and L:M ratio. The strength of the effect can be shown by the amount of luminance contamination required to counterbalance it. We have tested these predictions by measuring L:M ratio and pupil responses in a group of observers with a wide range of L:M ratio.

## 2. METHODS

### 2.1 Stimulus

A ColorDome ganzfeld (Diagnosys LLC, Lowell, MA, USA) was used to present the stimuli and to measure pupil responses. The device contains 4 classes of LED, tuned to red ( $\lambda_{\max}$ : 635nm, halfwidth at half-height: 10nm), amber ( $\lambda_{\max}$  590  $\pm$  8nm), green ( $\lambda_{\max}$  514  $\pm$  20nm) and blue ( $\lambda_{\max}$  460  $\pm$  15nm). Cone isolation was obtained using triple silent substitutions. The cone increments were presented on a steady background of 20cd.m<sup>-2</sup>, composed of red, amber, green and blue luminances of 8, 10, 1 and 1 cd.m<sup>-2</sup> respectively. The resultant CIE 1931 chromaticity of the background was x=0.424, y=0.223. This was somewhat more reddish than our previous studies and was used to maximize and equalize the available contrast of all 4 photoreceptor classes. An increment or decrement in the activation of a single photoreceptor class was achieved by calculating a brief change for each LED such that the stimulation of the other three photoreceptor classes remained constant. For full details, see the appendix in Maguire et al. [29]. Briefly, the triple silent substitutions were derived by multiplying the radiance of each of the 4 LED primaries by the cone and rod fundamentals [30, 31], and adjusting the output of the LEDs so that there was no overall change in activation of three of the four photoreceptor classes.

The central 3cm of the bowl was covered with a non-reflective matt black cloth disc so as not to stimulate the area of the retina with high levels of macular pigment [30, 32] which would affect the silent substitution calculation. This subtended a visual angle of 7° in diameter when the device was used in ganzfeld mode (i.e. with the subject's face against the front aperture)

### 2.2 L:M ratio

L:M ratio was estimated by presenting either an L-isolating or an M-isolating target in the ganzfeld, viewing this binocularly at 57 cm, and thus stimulating an oval retinal area 12° high and 15° wide, minus a 3° central patch. The target was flickered at a rate of 25Hz, maintaining mean hue and luminance at the background specified above, and reversing either side of this with a maximum cone contrast of 0.25. Using method of adjustment to determine flicker threshold, the observer made 5 settings. Contrast was adjustable in steps of 0.05 log units (1dB). L:M ratio was estimated by converting to linear contrast and dividing the L-value by the M-value [18]

### 2.3 Pupillometry

An Espion E<sup>2</sup> ophthalmic electrodiagnostic system (Diagnosys LLC, Lowell, MA, USA), modified by the manufacturer to record pupil diameter and position, was used to capture pupillary responses. A video camera is integrated into the ganzfeld, 5° above fixation. A LiveTrack eye movement recording interface (Cambridge Research Systems Ltd, Rochester, UK) provided continuous measurements of both pupil diameters at a rate of 100Hz. Calibration was conducted before each run using a standard pupil on a reference plate. A pair of infrared LEDs mounted in the ganzfeld provided accessory illumination of the pupil. Each new recording epoch was initiated by an external trigger from the Espion system which recorded the small fluctuations (of the order of 100s of  $\mu\text{m}$ ) in the pupils for the duration of the epoch. Around 10 to 30 epochs were averaged. Missed signals (due, for example, to blinks) were rejected manually retrospectively. Averages were stored for later analysis.

A recording epoch of 4000 ms was used, in which the change in activation lasted 1000 ms with an ISI of 3000 ms. The stimulus was viewed binocularly. Pupils were not dilated. 2 cycles were presented before the first recording was made. The data were stored as relative pupil diameter, using the diameter at the onset of each recording as the baseline. The experiments were conducted in a dimly-lit room (approximately 10 lux). Prior to the start of PLR recordings the subject adapted to the background for 1 minute.

Most of the data presented here used either L- or M-cone stimuli, with a Weber contrast of 0.25. In control studies we also modulated S-cones or rods, also with  $C=0.25$ , and, to compare with the conventional luminance response, we modulated all 4 receptor classes with either positive or negative contrast of 0.25.

### 2.4 Luminance bias

Small increments or decrements of luminance (luminance bias, LB) could be applied to all 4 primaries during the onset phase of the stimulus, with no change to the background. This was achieved by multiplying the luminance of each primary by the value of LB. Thus,  $LB = 1.0$  meant 'pure' cone isolation;  $LB < 1$  gave a luminance decrease and  $LB > 1$  an increase. See [5] for a detailed description of this approach. Each luminance bias run was prefaced with an unmodulated stimulus to establish baseline pupil diameter, followed by a trial in which all 4 photoreceptors were modulated with positive contrast of 0.25 (luminance onset stimulus). The luminance bias run itself then started with  $LB=1.55$ . Each successive trial decreased LB by 0.05, finishing with  $LB=0.45$  and a final luminance offset run in which all 4 photoreceptors were modulated with negative contrast ( $C = -0.25$ ). Thus a single run comprised 26 trials, each of between 10 and 30 stimuli, and took approximately 20 minutes to complete. The middle trial had a luminance bias of 1.0 ('pure' cone isolation).

### 2.5 Subjects

The study complied with the tenets of the declaration of Helsinki. Ethical approval was given by the University of Manchester ethics committee. 19 (4 male, 15 female) observers completed all runs. They were screened for colour vision deficiency (CVD) using HRR 4<sup>th</sup> edition (Richmond Products, Elgin, IL, USA) and had no known family history of CVD. They all gave their informed consent. Mean age ( $\pm$  s.d.) was 36.89 (16.26).

## 3. RESULTS

### 3.1 Individual photoreceptor responses

Figure 1 shows typical PLRs in two colour-normal subjects to all 4 classes of isolated photoreceptors. As we have previously described, the L-cone PLR is similar to the conventional luminance response, with a rapid onset and a slower offset response [1]. The

peak constriction is at around 800ms. Overall it is very small (less than 1mm) because these increments are presented on a light background with relatively low contrast, two properties forced by the use of silent substitution. The M-cone PLR shows the anomalous response which is the target of these investigations. In both subjects, the prominent constriction is to the offset of the cone stimulus. There is small response to stimulus onset in some observers and in a previous paper we called this a veridical response because it corresponds to the contraction that would occur if the pupil were acting conventionally [1]. S-cone stimulation produces a similarly paradoxical response, as previously reported by Woelders et al. [2]. Unlike the M-cone PLR, there is little evidence of a veridical onset constriction in these observers, but there may be individual variations. Finally, we also show the response to a rod-isolating increment, which is similar in its properties to the conventional light increment PLR.

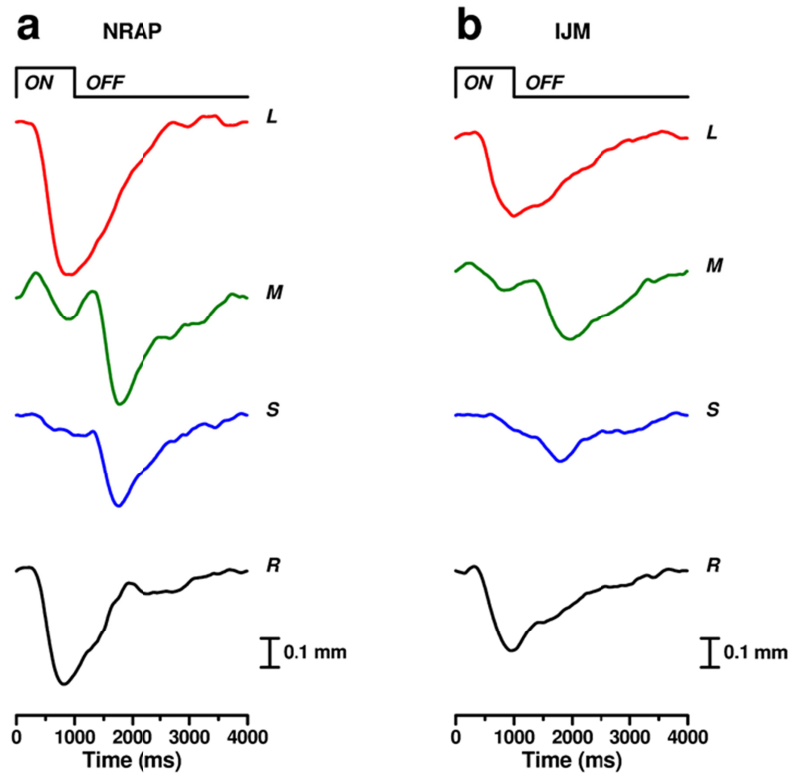


Figure 1 PLRs generated by isolated L-cone, M-cone, S-cone and rods. Note that the M-cone and S-cone response is primarily a contraction to the stimulus offset. PLR is relative to the diameter at time = 0.

### 3.2 L:M ratio

L:M ratio varied between 1.06 and 5.31 in our group of observers. Note that low ratios are quite well represented compared with other studies [e.g. 21]. This may be linked to the fact that some (n=8) subjects were Chinese; non-Caucasian individuals are likely to have lower L:M ratios [33].

### 3.3 Luminance bias

All 19 subjects completed the entire run of luminance bias PLRs using L and M-cone isolating stimuli in separate runs on the same day. Figure 2 shows a full set of data for three selected subjects, one with an L:M ratio close to the CIE standard observer (ER, 2.05), one with a high L:M ratio (IJM, 5.31) and one with L:M ratio approaching unity (XL, 1.17). Each subject produced a constriction to the onset of the luminance increment and to the offset of the luminance decrement (top and bottom rows). With  $LB = 1$  (cone isolation), all three subjects had a PLR showing constriction to the L-cone increment.

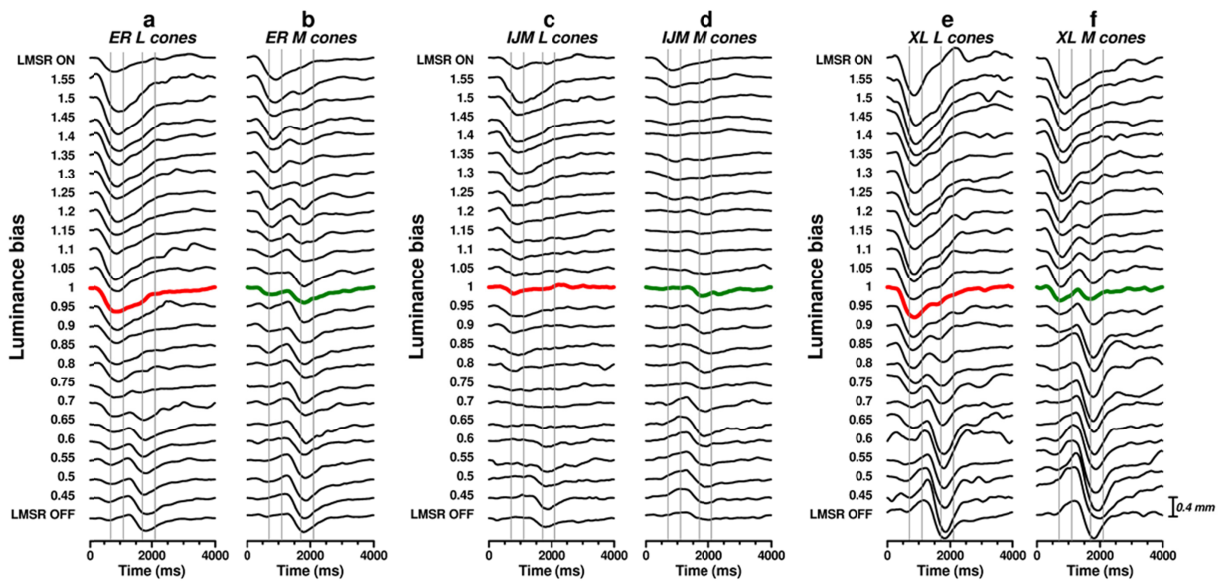


Figure 2 Pupillary light reflex responses for a range of luminance bias values either side of pure L- or pure M-cone stimulation. The top and bottom traces (labelled LMSR on and off) were recorded using either positive ('on') or negative ('off') contrast of 0.25 for L, M and S cones and rods. Data from 3 subjects with widely varying L:M ratios are shown. ER = 2.05 (a and b). IJM = 5.31 (c and d). XL = 1.17 (e and f). Responses when  $LB = 1$  are highlighted. PLR is plotted relative to the diameter at stimulus onset (time = 0). The vertical lines mark the windows which were used to calculate relative amplitude of onset and offset responses (see text).

The M-cone increment elicited a totally different sequence of responses that appears to vary systematically between observers. For  $LB = 1$ , the subject with L:M ratio of about 2 showed a classical anomalous PLR which was dominated by the offset but did show a veridical response to onset of relatively low amplitude. The subject with L:M ratio of approximately unity also showed a paradoxical constriction to stimulus offset, but the response to the onset is of higher magnitude. We refer to this as a weak version of the phenomenon. In fact, for this subject, inspecting the PLR for LB values slightly greater than 1.0 (1.05, 1.1 and 1.15) illustrates that the pupil response is close to being conventional which is not the case for the other observers. At this magnification the data for IJM suggests the

veridical response was totally absent at LB = 1.0 but this is misleading. In fact there is a small veridical response from this observer's pupil, as illustrated in Figure 1.

### 3.4 LB balance point as a function of L:M ratio

To gauge the strength of the paradoxical M-cone response, we assume it is proportional to the luminance bias required to balance the onset and offset responses. We call this the balance point. This is the LB at which there is equal pupillary constriction to both onset and offset. The absolute amplitude of the onset and offset responses was measured by calculating the minimum relative PD in two temporal windows, 700-1100ms for the onset and 1700-2100ms for the offset constriction, and taking their ratio. To find the balance point we constrained the ratios between 0 and 2, fitted an S-shaped curve to the data using logistic regression and calculated from this the value of LB which gave an on-off ratio of 1.0. The full derivation is shown in figure 3 for the same three observers as Figure 2. This approach allows us to concentrate on the region of interest (the transition from on- to off-dominance) without distraction from the more extreme values.

It is clear from figure 3 that the balance point is markedly different for these three observers with widely differing L:M ratio. These data suggest that lower L:M corresponds to a balance point that tends toward a luminance bias of 1.0.

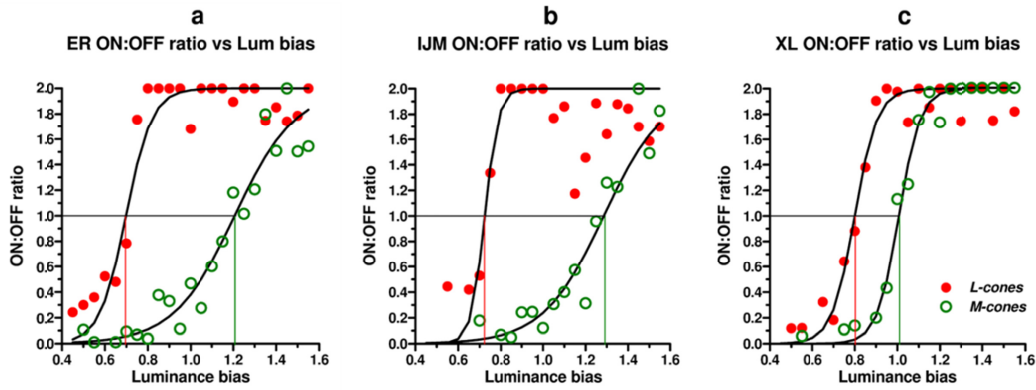


Figure 3 Logistic regression curves derived from luminance bias data. Horizontal line indicates the balance point. Vertical lines are extrapolations showing the method of calculating balance point. (a), (b) and (c) depict the same subjects as in figure 2.

Figure 4 shows both L and M balance points all 19 subjects, plotted as a function of log L:M ratio. These have been fitted with linear regression lines, whose equations are

$$BP_L = -0.16 \cdot \log(LMR) + 0.81$$

$$BP_M = 0.43 \cdot \log(LMR) + 0.99$$

where BP = balance point and LMR = L:M ratio. The three subjects whose full data have been shown are highlighted. Both graphs show an approximately linear relationship, with higher L:M ratios needing more luminance bias to counteract the effect in the M-cones. The L-cone data show a smaller and opposite effect using this analysis, in that increasing LMR leads to a higher level of luminance decrement being needed to counterbalance on and off responses. The L balance point is consistently below unity, which is what would be expected for a pure luminance response, as illustrated in Parry et al. [5].



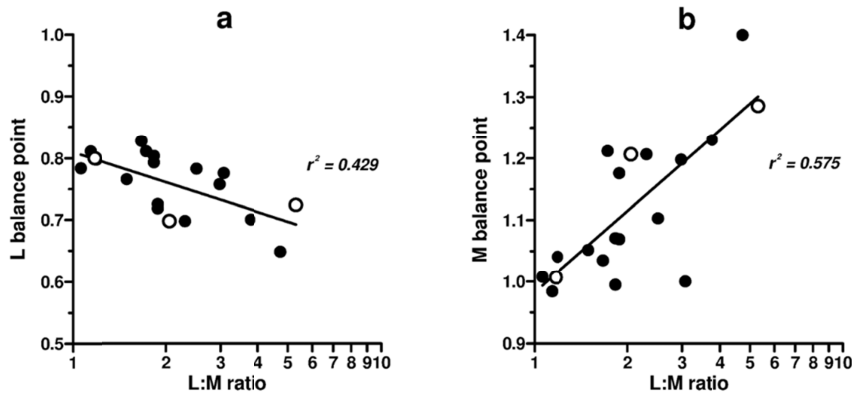


Figure 4 Balance point for (a) L-cone and (b) M-cone (right) stimuli vs L:M ratio. The open symbols depict the three subjects whose full data are shown in figures 2 and 3.

## 4. DISCUSSION

### 4.1 M-cone driven pupillary responses and the LB manipulation

In order to evaluate the strength of the paradoxical pupillary M-cone response in different individuals PLRs have been obtained from equal steps of luminance bias (LB) either added to or subtracted from cone isolating stimuli. The objective was to identify a particular luminance either side of the pure M-cone-isolating stimulus where the pupil responded ambivalently. This means that the pupil constricts equally strongly to both the onset and offset of the stimulus. At that balance point we argue that the strong inhibitory signal in the offset stimulus is compensated by the additional luminance in the LB, resulting in a constriction to the stimulus onset. If it is necessary to add substantial amounts of LB to induce this response, this implies that strong inhibitory signals are driving the pupil, essentially restricting its contraction to stimulus onset. An example might be subject IJM who has a relatively high LM ratio of 5.3 and for whom a balance point of 1.4 was derived. In contrast, for participant XL a relatively small amount of LB generated a prominent constriction to M-cone stimulus onset. Here the analysis yielded a value of 1.1 for the balance point, strongly suggesting that the inhibitory constriction-to-onset signal was relatively weak and easily overcome. This subject has L:M ratio of 1.17.

The wide range of L:M ratios in our study population allowed us to test the generality of these observations. Three examples of M- and L- cone LB sequences, representing the range of L:M ratios tested, are presented in figure 3 and there are clear and systematic differences between them. The M-cone LB sequence for observer XL is characteristic of all those with low L:M ratio. They invariably showed a strong, constriction-to-onset, veridical response and, with the addition of modest amounts of LB, this veridical constriction was increased so it became equal in amplitude to the paradoxical dilation induced by the offset. The opposite applies to observers with higher L:M ratio, for whom IJM and ER are representative. They require substantial amounts of additional LB in their M-cone stimulus for the M-cone onset to generate a contraction of the pupil. The pupils of these observers appear to be under stronger inhibitory influences than those with low L:M ratio.

This is consistent with the prediction in the introduction and the tentative hypothesis advanced in Murray et al. [1]. The L:M ratio of the observer acts as a strong influence on the existence and robustness of the anomalous paradoxical pupil response. We have also posited that the brightness and ERG versions of this effect are attributable to the same phenomenon

[3-5]. There is no compelling reason to suppose that the mechanisms might be different, but we are currently formally testing this. Figure 4 broadly confirms this relationship for the 19 subjects recruited to the study. When plotted on a log x-axis, L:M ratio is strongly linearly related to the M-cone balance point ( $r^2 = 0.561$ ;  $p < 0.01$ ).

#### *4.2 A putative mechanism for paradoxical M-cone pupil responses*

The retinal circuitry controlling cone opponency is composed of midget ganglion cells that use centre-surround receptive fields to antagonistically combine L and M cone signals. The centre of the receptive field is driven by individual input from either L or M cones via midget bipolars. It has long been thought that the ganglion cell inhibitory surround is derived from neighboring cones via H1 horizontal cells [6]. H1 cells receive convergent input from L and M cones, and the relative L and M contrast gains are determined simply by the relative numbers of L and M cones in the H1 cell's receptive field [34]. This was confirmed in a more recent study [35], in which pharmacological agents were used to investigate the receptive field surround. Both cone types always contributed to the surround, the physiological weight of the L and M cone inputs matching almost perfectly the cone ratio. If we assume human inhibitory surround is also derived from H1 cells then we can expect particularly strong inhibitory responses in L-cone rich retinæ, thus accounting for strong M-cone pupil paradox in observers with high L:M ratio.

#### *4.3 Pupillary control*

It has been widely reported that the pupil is under the control of a rich variety of stimulus properties such as spatial composition, motion and colour [e.g. 36]. Kimura and Young [26] showed pupil responses to be composed of different types of spectral responses and went on to develop a chromatic cancellation method. This revealed that pupillary constrictions generated with a red flash could be nulled by a simultaneous green flash and that these responses could be modelled with a cone opponent function whereby L cones were 1.58x more influential than M-cones [37]. They later described sustained pupillary constrictions that were partially mediated by L- and M- cone opponent interactions [38]. In the latter paper they speculated about the extent to which their responses might have been influenced by intrinsically photosensitive Retinal Ganglion Cells (ipRGCs). It is, on the face of it, a puzzle that the phenomenon we describe was not clearly evident in these studies. Two factors may be at play. Firstly, silent substitution is a somewhat artificial process in which the photoreceptors are effectively decoupled and, as we have speculated, this may emphasise the effect. Secondly, most of the subjects in these studies were Asian (personal communication from E. Kimura) and, as we previously mention, may well therefore have had low L:M ratio. Other work has shown a hint of the phenomenon. When investigating blind-sight, Barbur et al [27] recorded a constriction to the offset of their green colour opponent stimulus. This is likely to be a manifestation of the paradoxical effect described here. Colour opponent stimulation will of course contain contributions from both L and M cone mechanisms, and it seems plausible that the green offset related to the dominance by M-cones. We are investigating this in a separate study

The subtleties of the way pupil responses are shaped by ipRGCs are well documented [39-41]. Park and McAnany [42] have identified the profound differences between conventional and melanopsin-mediated pupillary responses. The latter are obtained primarily from bright, large, short-wave stimuli, occur in a time window 5-7 s after the stimulus offset and have a much slower time course than photoreceptor-based responses. There are compelling reasons for asserting that there is no significant melanopic influence over the observations reported here. We pointed out in our previous paper [1] that the melanopic contrast was some two orders of magnitude less than the M- and L cone contrast. Perhaps more crucially, in

previous papers we reported conventional ERGs from protanopes indicating that their M-cones perform normally when not linked to L cones by opponent mechanisms [3, 4]. It is presumed that our protanopic observers had normal melanopic function and that the ERG paradoxical response has the same basis as that of the PLR. The inverted M-cone PLR reported by Woelders et al. [2] is described in terms of its apposition to melanopic PLR. This is the case but it seems almost certain that the two sets of responses are unrelated.

#### ***4.4 Conclusion***

It is clear that observers with particularly L-cone rich retinae exhibit a PLR that constricts strongly to the offset rather than the onset of a M-cone isolating stimulus. In observers with low L:M the PLR to the onset and offset M-cone stimulation is similar indicating lower levels of inhibitory signals to the onset stimulation in these observers.

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#### **Disclosures**

The authors declare no conflicts of interest.

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