

## Using digital photography to study animal coloration

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In understanding how visual signals function, quantifying the components of those patterns is vital. With the ever-increasing power and availability of digital photography, many studies are utilizing this technique to study the content of animal colour signals. Digital photography has many advantages over other techniques, such as spectrometry, for measuring chromatic information, particularly in terms of the speed of data acquisition and its relatively cheap cost. Not only do digital photographs provide a method of quantifying the chromatic and achromatic content of spatially complex markings, but also they can be incorporated into powerful models of animal vision. Unfortunately, many studies utilizing digital photography appear to be unaware of several crucial issues involved in the acquisition of images, notably the nonlinearity of many cameras' responses to light intensity, and biases in a camera's processing of the images towards particular wavebands. In the present study, we set out step-by-step guidelines for the use of digital photography to obtain accurate data, either independent of any particular visual system (such as reflection values), or for particular models of nonhuman visual processing (such as that of a passerine bird). These guidelines include how to: (1) linearize the camera's response to changes in light intensity; (2) equalize the different colour channels to obtain reflectance information; and (3) produce a mapping from camera colour space to that of another colour space (such as photon catches for the cone types of a specific animal species). © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, 90, 211–237.

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### INTRODUCTION

Investigations into the adaptive functions of animal coloration are widespread in behavioural and evolutionary biology. Probably because humans are 'visual animals' themselves, studies of colour dominate functional and evolutionary investigations of camouflage, aposematism, mimicry, and both sexual and social signalling. However, with advances in our knowledge of how colour vision functions and varies across species, it becomes increasingly important to find means of quantifying the spatial and chromatic properties of visual signals as they are perceived by other animals or, at the very least, in a manner independent of human perception. This is nontrivial because colour is

not a physical property, but rather a function of the nervous system of the animal perceiving the object (Newton, 1718: 'For the rays, to speak properly, are not coloured'; Endler, 1990; Bennett, Cuthill & Norris, 1994). One way to produce an objective measure of the properties of a colour signal is to measure surface reflectance using spectrophotometry, which provides precise information on the intensity distribution of wavelengths reflected (Endler, 1990; Zuk & Decruyenaere, 1994; Cuthill *et al.*, 1999; Gerald *et al.*, 2001; Endler & Mielke, 2005). Reflectance data can also be combined with information on the illuminant and the photoreceptor sensitivities of the receiver (and, if available, neural processing) to model the colours perceived by nonhuman animals (Kelber, Vorobyev & Osorio, 2003; Endler & Mielke, 2005). However, conventional spectrometers provide only point samples, and to characterize adequately the colour of a heterogeneous object requires multiple samples across an

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appropriately designed sampling array, such as multiple transects or prespecified regions (Cuthill *et al.*, 1999; Endler & Mielke, 2005). This not only has a cost in terms of sampling time, but also the information about spatial relationships between colours then needs to be reconstructed from the geometry of the sampling array (Endler, 1984) and the spatial resolution is generally crude. Spectrometry also usually requires a static subject, either because of the need to sample an array or because the measuring probe often needs to be close to or touching the colour patch, a particular problem in the field or with delicate museum specimens. Focusing optics can obviate the need for contact with the animal or plant and offer a degree of 'remote sensing' (Marshall *et al.*, 2003; Sumner, Arrese & Partridge, 2005), but this approach is rare.

An alternative to spectrometry is photography, which has a long history of use in studies of animal coloration (Thayer, 1896, 1909; Cott, 1940; Tinbergen, 1974; Pietrewicz & Kamil, 1979) but is becoming increasingly used because of the flexibility and apparent precision that digital imaging provides. Colour change in the common surgeonfish (Goda & Fujii, 1998), markings in a population of Mediterranean monk seals (Samaranch & Gonzalez, 2000), egg crypsis in blackbirds (Westmoreland & Kiltie, 1996), the role of ultraviolet (UV) reflective markings and sexual selection in guppies (Kodric-Brown & Johnson, 2002), and the functions of primate colour patterns (Gerald *et al.*, 2001) comprise a few recent examples. Digital photography bears many advantages over spectrometry, particularly in the ability to utilize powerful and complex image processing algorithms to analyse entire spatial patterns, without the need to reconstruct topography from point samples. More obviously, photographing specimens is relatively quick, allowing rapid collection of large quantities of data, from unrestrained targets and with minimal equipment. Imaging programs can be used to obtain various forms of data, including colour patch size and distribution measures, diverse 'brightness' and colour metrics, or broadband reflection values (such as in the long-, medium-, and short wavebands). Video imaging can provide temporal information too. Digital technology also has the potential for manipulating stimuli for use in experiments, with the most impressive examples being in animations within video playback experiments (Künzler & Bakker, 1998; Rosenthal & Evans, 1998), although there are problems with these methods that need to be understood (D'Eath, 1998; Fleishman *et al.*, 1998; Cuthill *et al.*, 2000a; Fleishman & Endler, 2000).

Digital photography is increasingly incorporated into many studies of animal coloration due to its perceived suitability for objectively quantifying colour

and colour patterns. However, many studies appear to be unaware of the complex image processing algorithms incorporated into many digital cameras, and make a series of assumptions about the data acquired that are rarely met. The images recorded by a camera are not only dependent upon the characteristics of the object photographed, the ambient light, and its geometry, but also upon the characteristics of the camera (Barnard & Funt, 2002; Westland & Ripamonti, 2004). Therefore, the properties of colour images are device-dependent, and images of the same natural scene will vary when taken with different cameras because the spectral sensitivity of the sensors and firmware/software in different cameras varies (Hong, Lou & Rhodes, 2001; Yin & Cooperstock, 2004). Finally, the images are frequently modified in inappropriate ways (e.g. through 'lossy' image compression; for a glossary of some technical terms, see Appendix 1) and 'off-the-shelf' colour metrics applied without consideration of the assumptions behind them. At best, most current applications of digital photography to studies of animal coloration fail to utilize the full potential of the technology; more commonly, they yield data that are qualitative at best and uninterpretable at worst. This present study aims to provide an accessible guide to addressing these problems. We assume the reader has two possible goals: (1) to reconstruct the reflectance spectrum of the object (maybe just in broad terms such as the relative amounts of long-, medium- and short-wave light; although we will also consider something more ambitious) or (2) to model the object's colour as perceived by a nonhuman animal. Because we are considering applications of the accessible and affordable technology of conventional digital colour cameras, we are primarily focused on the human-visible spectrum of *c.* 400–700 nm, but we also consider UV imaging and combining this information with that from a standard camera. Our examples come from an investigation of colour patterns on lepidopteran wings, and how these might be viewed by avian predators. This is a challenging problem (birds are potentially tetrachromatic and have an UV-sensitive cone type; Cuthill *et al.*, 2000b), yet it is both tractable and informative, because much of the avian colour world overlaps with ours and birds are the focal organisms in many studies of animal coloration (whether their sexual signals, or the defensive coloration of their prey).

## CONCEPTUAL BACKGROUND

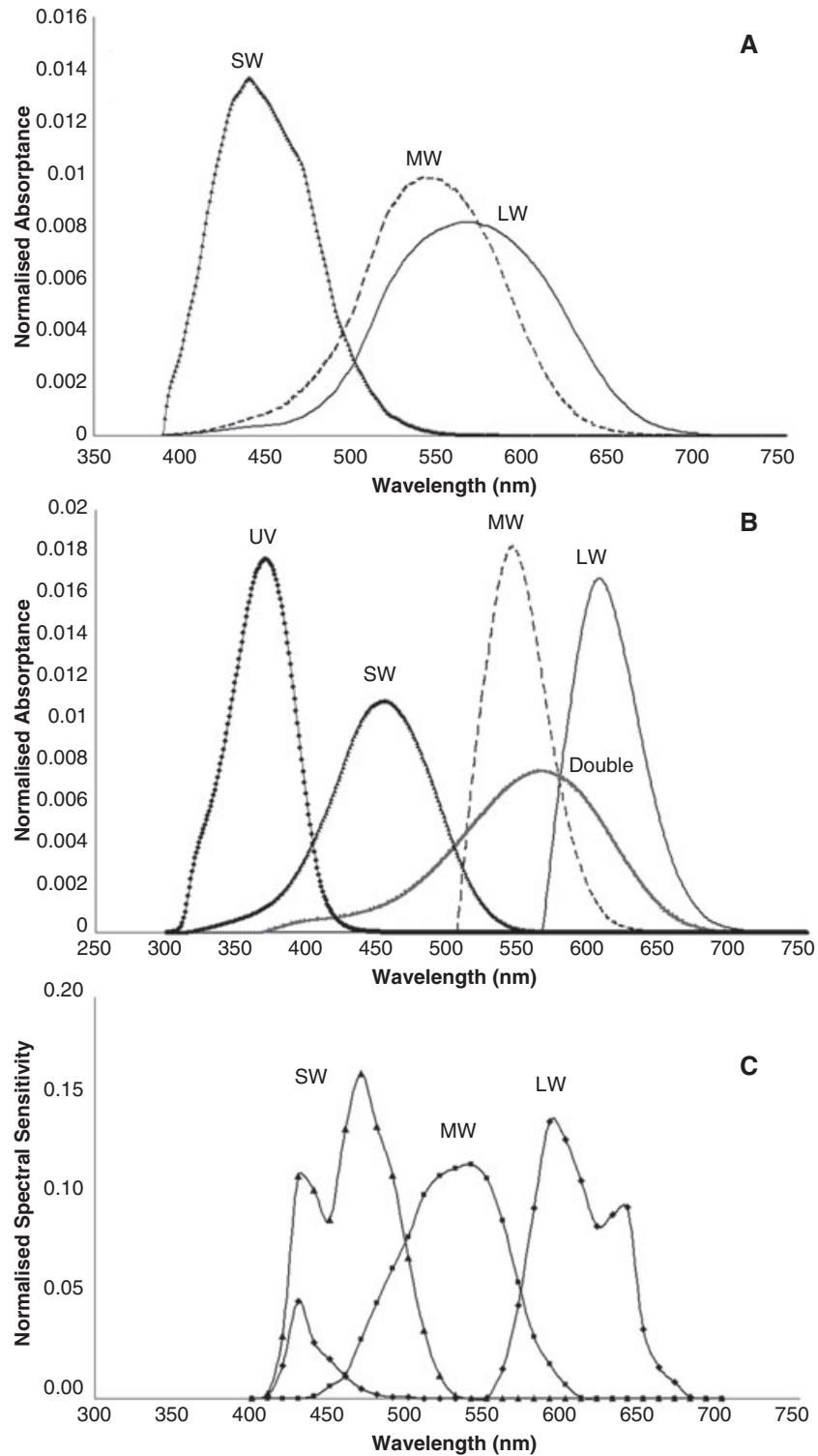
The light coming from a point on an object, its radiance spectrum, is a continuous distribution of different intensities at different wavelengths. No animal eye, or camera, quantifies the entire radiance spectrum at a given point, but instead estimates the intensity of light in a (very) few broad wavebands. Humans

and many other primates use just three samples, corresponding to the longwave (LW or 'red'), mediumwave (MW or 'green') and shortwave (SW or 'blue') cone types in the retina (Fig. 1A); bees and most other insects also use three samples, but in the UV, SW, and MW wavebands; birds and some reptiles, fish and butterflies use four samples (typically UV, SW, MW, and LW; Fig. 1B). A corollary of colour vision based on such few, broadband, spectral samples is that the colour appearance of an object can be matched, perfectly, by an appropriate mixture of narrow waveband lights ('primary colours') that differentially stimulate the photoreceptors. Three primary colours [e.g. red, green, and blue (RGB) in video display monitors] are required for colour matching by normally sighted humans. All that is required is that the mix of primary colours stimulates the photoreceptors in the same way as the radiance spectrum of the real object (without actually having to mimic the radiance spectrum *per se*). The additive mixing of three primaries is the basis of all video and cinematographic colour reproduction, and colour specification in terms of the amounts of these primaries, the so-called tristimulus values, lies at the base of most human colour science (Wyszecki & Stiles, 1982; Mollon, 1999; Westland & Ripamonti, 2004). That said, RGB values from a camera are not standardized tristimulus values and so, although they are easily obtained with packages such as Paintshop Pro (Corel Corporation; formerly Jasc Software) or Photoshop (Adobe Systems Inc.), simply knowing the RGB values for a point in a photograph is not sufficient to specify the colour of the corresponding point in the real object.

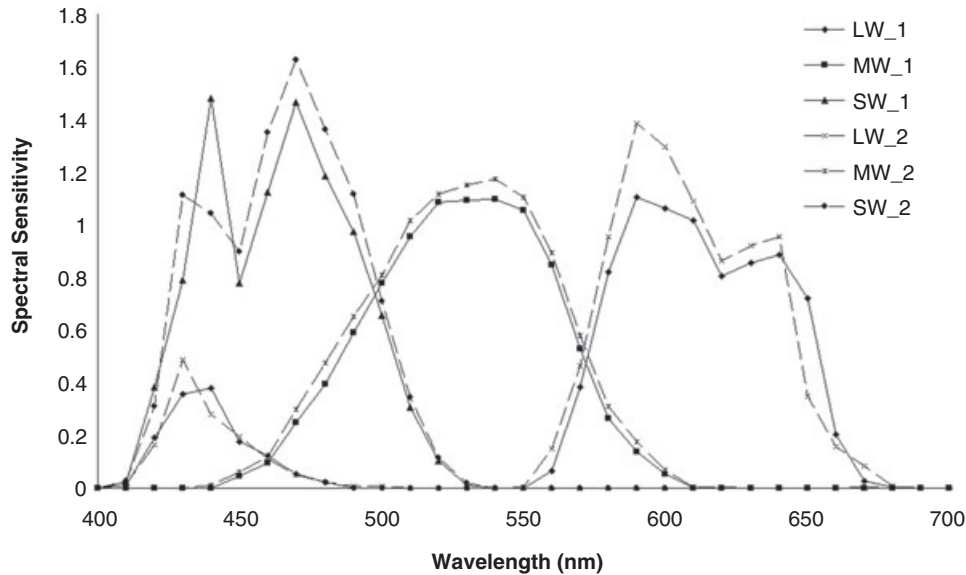
An over-riding principle to consider when using digital cameras for scientific purposes is that most digital cameras are designed to produce images that look good, not to record reality. So, just as Kodachrome and Fujichrome produce differing colour tones in 'analogue' film-based cameras, each film type having its own advocates for preferred colour rendition, the same is true of digital cameras. The values of R, G and B that are output from a camera need not be linearly related to the light intensity in these three wavebands. In technical and high-specification cameras they are, and the sensors themselves (the Charge Coupled Devices; CCDs) generally have linear outputs. By contrast, most cameras designed for non-analytical use have nonlinear responses (Cardei, Funt & Barnard, 1999; Lauzière, Gingras & Ferrie, 1999; Cardei & Funt, 2000; Barnard & Funt, 2002; Martinez-Verdú, Pujol & Capilla, 2002; Westland & Ripamonti, 2004). This is a function of post-CCD processing to enhance image quality, given the likely cross-section of printers, monitors, and televisions that will be used to view the photographs (these devices themselves having diverse, designed-in, nonlinearities; Westland & Ripamonti, 2004).

Most digital images will display well on most monitors because the two nonlinearities approximately cancel each other out. The first step in analysing digital images is therefore to linearize the RGB values.

Even with RGB values that relate linearly to R, G, and B light intensity, there is no single standard for what constitutes 'red', 'green', and 'blue' wavebands; nor need there be because different triplets of primary colours can (and, historically, have been) used in experiments to determine which ratios of primaries match a given human-perceptible colour (Mollon, 1999; Westland & Ripamonti, 2004). The spectral sensitivities of the sensors in a digital camera need not, and usually do not, match those of human visual pigments, as was the case with the Nikon 5700 Coolpix camera primarily used in this study (Fig. 1C). The RGB values in images from a given camera are specific to that camera. Indeed, the values are not necessarily even specific to a particular make and model, but rather specific to an individual camera, because of inherent variability in CCDs at the manufacturing stage (Fig. 2). One can, however, map the camera RGB values to a camera-independent, human colour space (and, under some circumstances, that of another animal) given the appropriate mapping information. Therefore, the mapping, through mathematical transformation, of the camera-specific RGB values to camera-independent RGB (or other tristimulus representation) is the second crucial step in obtaining useful data from a digital image. Furthermore, and often as part of the transformation step, it will usually be desirable to 'remove' variation due to the illuminating light. The camera measures R, G, and B radiance, which is the product of the reflectance of the object and the three-dimensional radiance spectrum illuminating the object (often approximated by the irradiance spectrum of the illuminant). The situation is rather more complex underwater, where the medium itself alters the radiance spectrum (Lythgoe, 1979) by wavelength-dependent attenuation. However, an object does not change colour (much) when viewed under a blue sky, grey cloud, or in forest shade, even though the radiance spectra coming from it changes considerably. This phenomenon of 'colour constancy', whereby the visual system is largely able to discount changes in the illuminant and recover an object's reflectance spectrum, is still not fully understood (Hurlbert, 1999), but equivalent steps must be taken with digital images if it is object properties that are of interest rather than the radiance itself. Many digital cameras allow approximations of colour constancy (white-point balancing) at the point of image acquisition; for example by selecting illuminant conditions such as skylight, cloudy, and tungsten. However, these settings are an approximation and, in practice, their effects



**Figure 1.** A, normalized absorbance (equal areas under curves) of human cones. Absorbance ( $N$ ) data from Dartnall, Bowmaker & Mollon (1983) converted to absorbance ( $P$ ) by the equation  $P = 1 - 10^{-NLS}$ , where  $L$  is the length of the cone (20  $\mu\text{m}$  from Hendrickson and Drucker, 1992), and  $S$  is specific absorbance,  $0.015/\mu\text{m}^{-1}$ . B, normalized absorbance (equal areas under curves) of starling cones to different wavelengths of light. From Hart, Partridge & Cuthill (1998). C, normalized spectral sensitivity (equal areas under curves) of the sensors in the Nikon 5700 Coolpix camera used in the present study. SW, shortwave; MW, mediumwave; LW, longwave; UV, ultraviolet.



**Figure 2.** A plot of spectral sensitivity of two Nikon 5700 cameras for the longwave (LW), mediumwave (MW), and shortwave (SW) channels. Even though the cameras are the same make and model, and were purchased simultaneously, there are some (albeit relatively small) differences in spectral sensitivity.

need to be eliminated because the effect of the illuminant itself needs to be 'removed'. Removing the effect of the light source characteristics can thus be coupled to eliminating any biases inherent in the camera's image processing (such as an over-representation of some wavelengths/bands to modify the appearance of the photograph; Cardei *et al.*, 1999; Finlayson & Tian, 1999; Lauzière *et al.*, 1999; Martinez-Verdú *et al.*, 2002). This is essential if accurate data representing the inherent spectral reflection characteristics of an animal's colour are to be obtained.

Many studies have used cameras to investigate animal colour patterns, but most fail to test their digital cameras to determine if all of the above assumptions are met and/or if the analysis yields reliable data (Frischknecht, 1993; Villafuerte & Negro, 1998; Wedekind *et al.*, 1998; Gerald *et al.*, 2001; Kodric-Brown & Johnson, 2002; Bortolotti, Fernie & Smits, 2003; Cooper & Hosey, 2003); for a rare exception, see Losey (2003).

We approach these problems in the sequence that a scientist would have to address them if interested in applying digital photography to a research question about biological coloration. This study focuses on obtaining data corresponding to inherent animal coloration, such as reflection data, and of obtaining data relevant to a given receiver's visual system. Either of these data types may be more suitable depending upon the research question. Reflection data does not assume specific environmental conditions or a particular visual system viewing the object, and so data can be compared across different specimens easily, even

when measured in different places. The lack of assumptions about the receiver's visual system, such as photoreceptor types, distributions, abundances, sensitivities, opponency mechanisms, and so on, means the data 'stand alone' and can be analysed as an inherent property of the animal or an object propagating the signal. This is useful if a researcher simply wishes to know if, for example, individual 'a' has more longwave reflection than individual 'b'. Removing illumination information coincides with evidence that many animals possess colour constancy. Conversely, simply taking reflection into account could be misleading if what one really wants to know is how a signal is viewed by a receiver. For example, if an individual possesses a marking high in reflection of a specific waveband, but the environment lacks light in that part of the spectrum or the receiver is insensitive to that waveband, the region of high spectral reflection will be unimportant as a signal. Therefore, it is often necessary to include the ambient light characteristics and, if known, information concerning the receiver's visual system. However, calculated differences in photon catches of various photoreceptor types (for example) between the different conditions do not necessarily lead to differences in perception of the signal, if colour constancy mechanisms exist. Furthermore, if reflection information is obtained, this may be converted into a visual system specific measure, either by mapping techniques, as discussed here, or by calculations with illuminant spectra and cone sensitivities. Therefore, although the present study deals with both types of measurements, we focus more on the task of

obtaining information about inherent properties of animal coloration.

We assume that images are stored to a precision of 8 bits in each colour channel, such that intensity is on a scale of 0–255; such ‘true colour’ images ( $2^8$  cubed, or >16 million colours) are the current norm. Although some studies have used conventional (nondigital) cameras to study animal coloration, we would advise against doing so. Although conventional film can be linearized, the corrections required from one batch of film to the next are likely to differ, even from the same manufacturer. Film processing techniques, such as scanning to digitize the images, are also likely to introduce considerable spatial and chromatic artefacts, which need to be removed/prevented before analysis.

### CHOOSING A CAMERA

We have mentioned the nonlinear response of many digital cameras and although we show (below) how linearization can be accomplished, nonlinearity is better avoided. Other than this, essential features to look for are (Table 1):

1. The ability to disable automatic ‘white-point balancing’. This is a software feature built into most cameras to achieve a more natural colour balance under different lighting conditions. The brightest pixel in any image is set to 255 for R, G, and B (i.e. assumed to be white). Obviously, for technical applications where the object to be photographed has no white regions, this would produce data in which the RGB values are inappropriately weighted.
2. A high resolution. The resolution of a digital image is generally limited by the sensing array, rather than the modulation transfer function of the lens. Essentially, the number of pixels the array contains determines resolution, with higher resolution cameras able to resolve smaller colour patches allowing more detail to be measured, or the same amount of relative detail measured from a further distance from the subject.

Also important is the Nyquist frequency (half that of the highest frequency spatial waveform), which is the highest spatial frequency where the camera can still accurately record image spatial detail; spatial patterning above this frequency results in aliasing, which could be a problem for patterns with a very high level of spatial detail (Efford, 2000). There is no set rule as to what a minimum level of pixels in an image should be; if it is possible to work in close proximity to the object, then even a 0.25-megapixel image may be sufficient. The problem is to avoid Nyquist limit problems, where the pixels need to be less than half the size of the smallest detail in the image that you are interested in. Each pixel on a digital camera sensor contains a light sensitive photodiode, measuring the intensity of light over a broadband spectrum. A colour filter array is positioned on top of the sensor to filter the red, green, and blue components of light, leaving each pixel sensitive to one waveband of light alone. Commonly, there is a mosaic of pixels, with twice as many green sensitive ones as red or blue. The two missing colour values for each individual pixel are estimated based on the values of neighbouring pixels, via so-called demosaicing algorithms, including Bayer interpolation. It is not just the number of pixels a camera produces (its geometrical accuracy) that matters, but also the quality of each pixel. Some cameras are becoming available that have ‘foveon sensors’, with three photodetectors per pixel, and can thus create increased colour accuracy by avoiding artefacts resulting from interpolation algorithms. However, due to the power of the latest interpolation software, colour artefacts are usually minor, especially as the number of pixels increases, and foveon sensors may have relatively low light sensitivity. Higher quality sensors have a greater dynamic range, which can be passed on to the images, and some cameras are now being produced with two photodiodes per pixel: one of which is highly sensitive to low light levels, the other of which is less sensitive and is used to estimate higher light levels without becoming saturated. A distinction should also be made between the

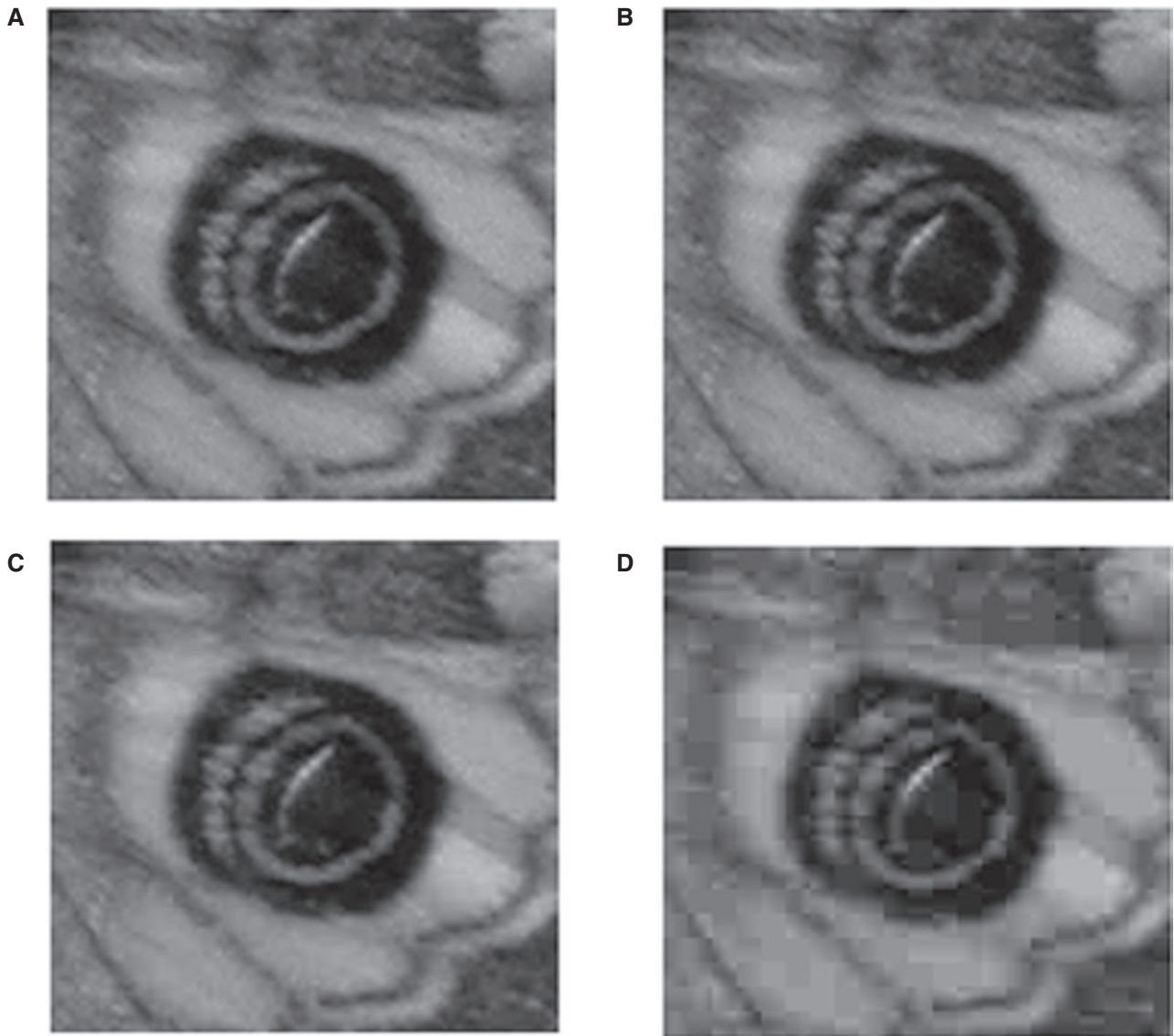
**Table 1.** Desirable characteristics when purchasing a digital camera for research

Attribute	Relative importance
High resolution (e.g. minimum of 5 megapixels)	Medium (depends upon the complexity/size of the object photographed)
Manual white balance control	High
Macro lens	Medium
Ability to save TIFF/RAW file formats	High
Manual exposure control	High
Remote shutter release cable capability	Low
Ability to change metering method	Medium
Optical zoom	Medium

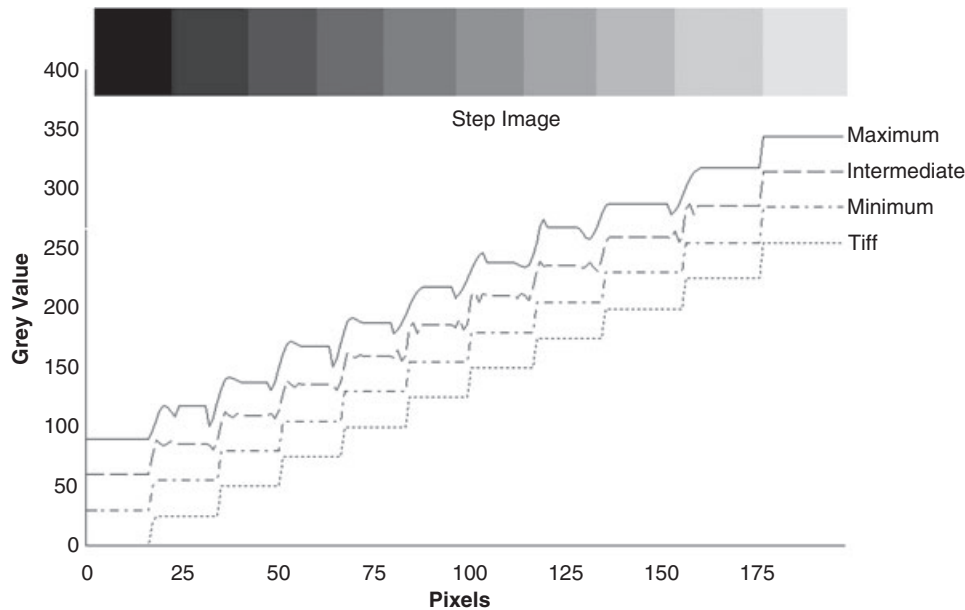
number of overall pixels and the number of effective pixels. A conventional 5 megapixel camera actually may output  $2560 \times 1920$  pixel images (4915 200 pixels) because some of the pixels in the camera are used for various measurements in image processing (e.g. dark current measurements).

3. The ability to store images as uncompressed TIFF (Tagged Image File Format) or RAW files. Some mid-range cameras allow storage as RAW files, others do not but often allow images to be saved as TIFF files. This is something to determine before purchasing a camera. Other file types, in particular JPEGs (Joint

Photographic Experts Group), are unsuitable because information is lost in the compression process. JPEG compression is of the 'lossy' type, which changes the data coming from the CCD array, and where the lost information cannot be recovered. This is often undetectable to the human eye, but introduces both spatial and chromatic artefacts in the underlying image data, particularly if the level of compression is high (for two simple illustrations, see Figs 3, 4). JPEGs compress both the colour and spatial information, with the spatial information sorted into fine and coarse detail. Fine detail is discarded first because this is what we are



**Figure 3.** Four images of the hind left spot on the emperor moth *Saturnia pavonia* illustrating the effects of compression on image quality. A, an uncompressed TIFF image of the original photograph. B, a JPEG image with minimal compression (10%). C, a JPEG image with intermediate compression (50%), which still appears to maintain the original structure of the image, but careful examination of the image's spatiochromatic content shows inconsistencies with the original TIFF file. D, a JPEG image with maximal compression (90%) showing severe spatial and chromatic disruption.



**Figure 4.** Grey values measured when plotting a transect across a grey scale step image with increasing values from left to right. Grey values start at 0 on the left of the series of steps and increase in steps of 25 to reach values of 250 on the right. Plotted on the graph are the values measured for images of the steps as an uncompressed TIFF file, and JPEGs with ‘minimum’ (10%), ‘intermediate’ (50%), and ‘maximum’ (90%) levels of compression. Values of 30, 60, and 90 have been added to the JPEG files with minimum, intermediate and maximum levels of compression to separate the lines vertically. Note that, as the level of compression increases, the data measured are more severely disrupted, particularly at the boundary between changes in intensity. In the case of complex patterns, the disruption to the image structure means that measurements at any point in the image will be error prone.

less sensitive to. For example, Gerald *et al.* (2001) used digital images to investigate the scrota of adult vervet monkeys *Cercopithecus aethiops sabaesus*. They saved the images as JPEG files but, because the level of compression of the files is not stated, it is impossible to assess the degree of error introduced. Camera manuals may state the level of compression used on different settings, and image software should also state the level of compression used when saving JPEG files. However, even if the level of compression is known, the introduction of artefacts will be unpredictable and so JPEG files should be avoided. Lossy compression is different from some other types of compression, such as those involved with ‘zipping’ file types, where all the compressed information can be recovered. Uncompressed TIFF files are loss-less, but TIFF files can be compressed in either lossy or loss-less ways, and, like JPEGs, TIFFs can be modified before being saved in other ways if the necessary camera functions are not turned off (such as white-point balancing). For most cameras, a given pixel on a CCD array has only one sensor type (R, G, or B), and interpolation is required to estimate the two unknown colour values of a given pixel. Both JPEGs and TIFF files undergo interpolation at the stage of image capture by the camera’s internal firmware, which cannot be turned off, and the

method is usually opaque to the user. Some cameras have the capacity to store RAW images. RAW files are those that are the direct product of the CCD array, and, unlike TIFFs or JPEGs which are nearly always 8-bit, RAW files are usually 12- or 16-bit. This means they can display a wider variety of colours and are generally linear because most CCDs are linear, and undergo none of the processing potentially affecting other file types. The RAW files from the camera in our study occupy approximately half of the memory of an uncompressed TIFF file because even though the TIFF file only retains 8-bits of information, it occupies twice the storage space because it has three 8-bit colour channels, as opposed to one 12-bit RAW channel per CCD pixel. However, before being useable as an image, RAW files must also go through interpolation steps in the computer software into which the files are read. Thumbnails of unprocessed RAW files in RGB format can be read into some software, but these are relatively useless, being only  $160 \times 120$  pixels in resolution, compared to  $2560 \times 1920$  pixels for the processed images. The conversion to another file type can proceed with no modification, just as would be the case if taking photos directly as uncompressed TIFF images. One problem with RAW files is that they can differ between manufacturers and even between camera



models, and so special software and/or 'plug-ins' may be needed, or the software provided by the manufacturer must be used, to convert the images to other file formats. Unfortunately, the interpolation process is rarely revealed by the manufacturer, and may introduce nonlinearities into the file. It is possible to write custom programmes to read RAW files into software programmes and this has the advantage that the user can then either use the RAW data directly or decide exactly what method should be used to interpolate the RAW file into a TIFF file. Once our RAW files had been processed by software supplied by the manufacturer, they had almost identical properties to the uncompressed TIFF files (the introduction of nonlinearities could be due to the software processing or a nonlinear CCD). Some imaging software should allow the RAW files to be processed into TIFFs without introducing nonlinearities. RAW files can also be converted into 16-bit TIFF files, which show higher accuracy than 8-bit TIFFs and may highlight extra detail. These 16-bit file types occupy approximately 30 Mb, so considerable storage space is needed to keep a large number of these files. However, relatively more unprocessed RAW files can be stored than TIFFs on a memory card.

4. The capacity for manual exposure control or, at the very least, aperture priority exposure. The calibration curve may vary with different aperture settings and focus distances so, to avoid the need for a large number of separate calibration estimates, it is more convenient to fix the aperture at which photographs are taken and work at constrained distances. If the aperture value is increased, more light from the edge of the lens is allowed through, and these rays usually do not converge on the same point as those rays coming through the centre of the lens (spherical aberration). This is especially true for colours near the edges of the human visible spectrum. By keeping the aperture constant and as small as possible (large F-numbers), this problem is unlikely to be significant.

5. The ability to take a remote shutter release cable (manual or electronic) to facilitate photography at long integration times (slow shutter speeds) when light levels are low.

6. Known metering characteristics. Many cameras have multiple options for light metering, such that the exposure is set dependent upon average intensity across the entire field imaged, only the intensity at the central spot, or one or more weighted intermediates. Knowing which area of the field in view determines exposure facilitates image composition.

7. Optical zoom can be useful, particularly if the level of enlargement can be fixed manually, so it can be reproduced exactly, if needed, each time the camera is turned on. Digital zoom is of no value because it is merely equivalent to postimage-capture enlargement and so does not change the data content of the area of interest.

8. Good quality optics. One problem with lenses is chromatic aberration, in which light of different wavelengths is brought to a focus in a different focal plane, thus blurring some colours in the image. This can be caused by the camera lens not focusing different wavelengths of light onto the same plane (longitudinal chromatic aberration), or by the lens magnifying different wavelengths differently (lateral chromatic aberration). Párraga, Troscianko & Tolhurst (2002) tested camera lenses of the type in our Nikon camera, by taking images in different parts of the spectrum through narrowband spectral filters and verified that the optimal focus settings did not vary significantly, meaning that the lenses did not suffer from this defect. Narrow bandpass filters selectively filter light of specific narrow wavebands (e.g. from 400 to 410 nm). Using a set of these filters enables images to be obtained where the only light being captured is in a specific waveband. Other lenses may not be as good, especially if they have a bigger optical zoom range. Therefore, aside from the requirement to produce images free from problems such as spherical aberration, the most important issue is to minimize chromatic aberration. As with Párraga *et al.* (2002), a good test for this is to take images of a page of text under white light through narrowband red and blue filters without changing the focus (this requires manual focus). If there is no chromatic aberration, then both images should be equally sharp. A more formal test is to measure the Fourier spectrum of the two images; if there is a good correction for chromatic aberration the two spectra should be the same. Furthermore, Hong *et al.* (2001) noted that, in some camera lenses, light is not uniformly transmitted across its area, with the centre of the lens transmitting more light. This would result in the pixels in the centre of the image being over-represented in terms of intensity. This potential problem should be tested for. Losey (2003) also found that the edges of images were slightly darker. In some situations, a good macro lens is also highly desirable because this allows close up images of complex patterns to be obtained. Without a macro lens, it may not be possible to move the camera close enough to resolve complex patterns. Some cameras even come with a 'super' macro lens, such as the Fujifilm FinePix S7000, which allows photographs to be taken up to 1 cm from the object.

9. The capacity to take memory cards of high capacity. TIFF files are very large (c. 15 Mb for an image 2560 by 1920 pixels), so that a 512 Mb card that can store over 200 medium-compression JPEGs will only store 34 TIFFs.

## IMAGE COLOUR VALUES

The colour values to be calculated and used in any analysis are stored as RGB values in TIFF files auto-

matically when a camera saves an image or when a file is converted into a TIFF image from its RAW file format and, if 8-bit, this is on a scale of 0–255. The camera or computer conversion software may have the option to save the image as either 8-bit or 16-bit, but 8-bit is currently more standard. The steps that follow to calculate values corresponding to, for example, reflection or photon catches are spelt out below. If adjusting an image with a standard or set of standards to recover reflectance, then the standards should have a flat reflectance spectrum (i.e.  $R = G = B$ ); therefore, the image values are adjusted so that  $R = G = B$  in the linearized picture. This will give an image in which the pixels have the correct relative spectral reflectance. At this point, a crucial issue to emphasize is that many image software programmes offer the option to convert values into other colour spaces, such as HSB (three images corresponding to hue, saturation, and brightness). Conversions such as HSB should be avoided and we strongly advise against this type of conversion. HSB is a human-vision-specific colour space, and even in terms of human vision, it is unlikely to be accurate; a more widely used and well tested colour space for humans is the Commission Internationale de l'Éclairage (CIE) Laboratory colour space, which may in some cases be appropriate. There are numerous pitfalls with using methodological techniques based on human vision to describe animal colours (Bennett *et al.*, 1994; Stevens & Cuthill, 2005).

### SOFTWARE

One of the biggest advantages of using images to analyse coloration is the existence of a huge number of flexible and powerful software programmes, coupled with the option to write custom programmes in a variety of programming languages. Some of the programmes available to deal with image processing are standard and quite affordable, such as Paintshop Pro or Photoshop, which can be used for a range of simple tasks. However, there are a range of other options available, including the popular freeware programmes such as the open-source image editor GIMP and the Java-based (Sun Microsystems, Inc.; Efford, 2000) imaging programme 'Image J' (Rasband, 1997–2006; Abramoff, Magalhães & Ram, 2004), with its huge variety of available 'plugins', written by various people for a range of tasks. Image J also permits custom programmes written in the language Java to accompany it. For example, a plugin that we used, called 'radial profile', is ideal for analysing lepidopteran eyespots, and other circular features. This works by calculating the normalized intensities of concentric circles, starting at a central point, moving out along the radius. Figure 5 gives an example of this plug-in, as used to

analyse an eyespot of the ringlet butterfly *Aphantopus hyperantus*.

The programme MATLAB (The Mathworks Inc.) is also an extremely useful package for writing calibrations and designing sophisticated computational models of vision. This is a relatively easy programming language to learn, is excellent for writing custom and powerful programmes, and, due to its matrix manipulation capabilities, is excellent for dealing with images (digital images are simply matrices of numbers). MATLAB can also be bought with a range of 'toolboxes' that have numerous functions already written for various tasks, including image processing, statistics, and wavelet transformations. MATLAB has available an Image Processing Toolbox with a range of useful functions (Hanselman & Littlefield, 2001; Hunt *et al.*, 2003; Gonzalez, Woods & Eddins, 2004; Westland & Ripamonti, 2004).

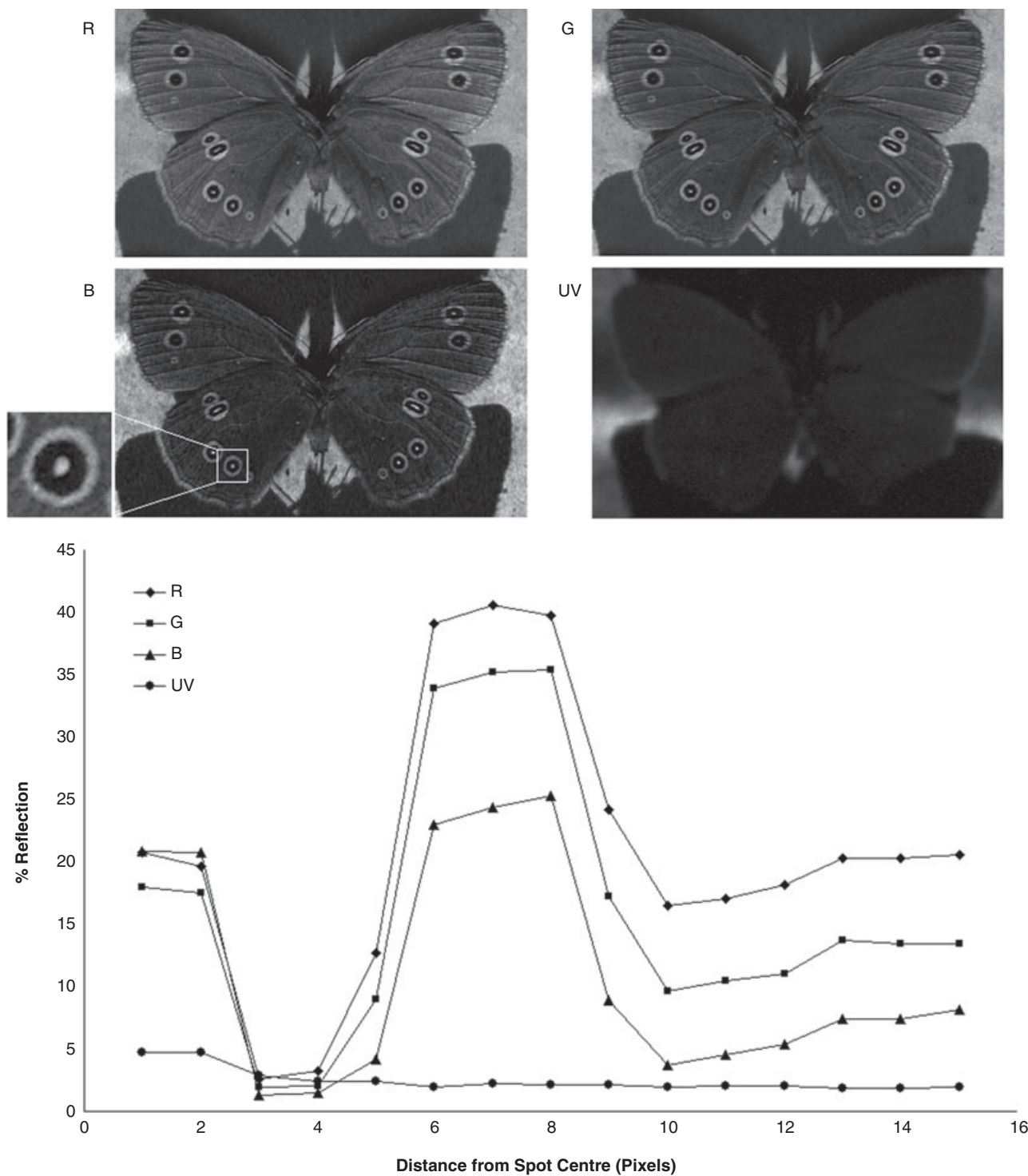
### HOW FREQUENTLY SHOULD CALIBRATIONS BE UNDERTAKEN?

The frequency with which calibrations should be undertaken depends upon the specific calibration required. For example, determining the spectral sensitivity of a camera's sensors need only be performed once because this should not change with time as long as the lens on the camera is not changed, in which case recalibration may be needed. Additionally, the calculation of the camera's response to changing light levels and the required linearization need only be performed once because this too does not change with time. However, if calculating reflection, the calibration needs to be performed for each session/light setup because the light setup changes the ratio between the LW, MW, and SW sensors.

### CALIBRATING A DIGITAL CAMERA

There are several steps that should be followed when wishing to obtain values of either reflection or data corresponding to an animal's visual system. To obtain values of reflection:

1. Obtain images of a set of reflectance standards used to fit a calibration curve.
2. Determine a calibration curve for the camera's response to changes in light intensity in terms of RGB values.
3. Derive a linearization equation, if needed, to linearize the response of the camera to changes in light intensity, based on the parameters determined from step 2.
4. Determine the ratio between the camera's response in the R, G, and B channels, with respect to the reflectance standards, and equalize the response of



**Figure 5.** Results from a radial profile analysis performed upon one eyespot of the ringlet butterfly *Aphantopus hyperantus*, illustrating the high percentage reflectance values obtained for the centre of the spot and the ‘golden’ ring further from the centre, particularly in the red and green channels, and the lack of an eyespot in the ultraviolet (UV).

the different colour channels to remove the effects of the illuminating light and any biases inherent in the camera's processing.

If data corresponding to an animal's visual system is required (such as relative photon catches):

1. Obtain photographs of reflectance standards through a set of narrow band-pass filters, at the same time as measuring the radiance with a spectrophotometer.
2. Determine the linearity of the camera's response to changing light levels and, if necessary, derive a linearization. Furthermore, using radiance data and the photographs through the band-pass filters, determine the spectral sensitivity of the camera's different sensor types.
3. Using data on the spectral sensitivity of the camera's sensors, and the sensitivity of the animal's sensors to be modelled, produce a mapping based on the response to many different radiance spectra between the two different colour spaces.

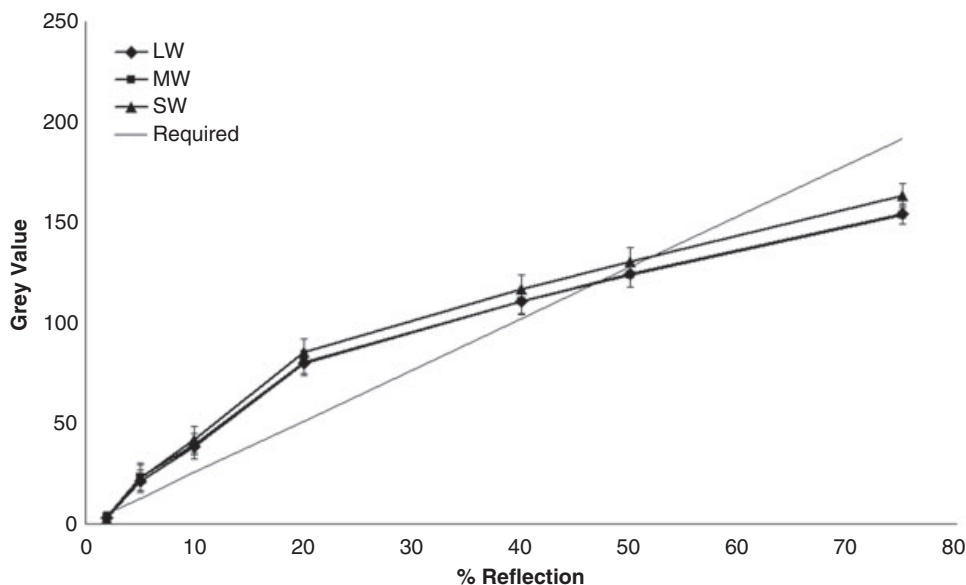
These different steps are discussed in detail below.

### LINEARIZATION

If a set of grey reflectance standards is photographed and then the measured RGB values are plotted against the nominal reflectance value, the naïve expectation would be of a linear relationship (Lauzière *et al.*, 1999). One might also expect the values

obtained for each of the three colour channels to be the same for each standard because greys fall on the achromatic locus of  $R = G = B$  (Kelber *et al.*, 2003). However, as mentioned previously, many cameras do not fulfil such expectations, and they did not for the Nikon 5700 Coolpix camera that we used in our study (Fig. 6; see Appendix 2). A different nonlinear relationship between grey value and nominal reflection for each colour channel requires that the linearizing transformation must be estimated separately for each channel. Also, it means that an image of a single grey reflection standard is insufficient for camera calibration; instead a full calibration experiment must be performed.

We used a modification of the linearization protocols developed by Párraga (2003) and Westland & Ripamonti (2004). The first step is to photograph a range of standard greyscales of known reflectance value. Westland & Ripamonti (2004) used the greyscale of the Macbeth ColorChecker chart (Macbeth, Munsell Color Laboratory). In the present study, because we required reflection standards suitable for UV photography (see below), we used a set of Spectralon diffuse reflectance standards (Labsphere Inc.). These standards, made of a Teflon microfoam, reflect light of wavelengths between 300 nm and 800 nm (and beyond) approximately equally, and are one of the most highly Lambertian substances available over this spectral range. The standards had nominal percentage reflection values of 2%, 5%, 10%, 20%, 40%,



**Figure 6.** The relationship between the grey scale value measured for a set of seven Spectralon reflectance standards from raw digital TIFF file images and the nominal reflection value, showing a curved relationship for the R, G, and B data. The 'required' line illustrates values that should be measured if the camera's response was linear and the three channels equally stimulated. LW, longwave; SW, shortwave; MW, mediumwave.

50%, and 75%. If the object of the study, as in Westland & Ripamonti (2004: chapter 10) and the present study, is to recover reflectance data from the images, then the nature of the illuminant, as long as it is stable over time (Angelopoulou, 2000) and of adequate intensity in all wavebands, is irrelevant. We used a 150-W Xenon arc lamp (Light Support), which was allowed to warm up and stabilize for 1 h before the calibration exercise, and then tested for stability before and after the calibration. In Párraga (2003), the goal was to recover spectral radiance; thus, at the same time as photographing the standards, the radiance of each greyscale patch was measured using a spot-imaging telespectroradiometer (TopCon Model SR1, Calibrated by the National Physical Laboratory). After that, each sensor's grey level output was plotted against a measure of the total spectral radiance that stimulated it, at various shutter speeds. Because radiance, unlike reflectance, varies with the illuminant, Párraga (2003) repeated the calibration process under a variety of lighting conditions. If recovering radiance is the objective, it is important to determine the calibration curve appropriate to the conditions under which the research photographs will be taken. Objects will give problems of metamerism if their reflectance spectra are 'spiky' or, at least, very uneven. It is therefore important to check the linearization calibration works for objects from the same class as those being measured.

The next step is to determine the function relating the intensity values (0–255) for each of the RGB sensors to true reflection, or radiance, as appropriate, as measured spectrometrically. Many studies describe power functions of the same family as those relating intensity to voltage in cathode ray tube monitors; these are so-called gamma functions of the type: Output = constant  $\times$  (input) <sup>$\gamma$</sup> . For this reason, the linearization process is sometimes referred to as 'gamma correction'. The term gamma function means different things in film photography, digital photography, and algebraic mathematics, and so is a potentially confusing term that is best avoided. Because the response of the camera's sensors is likely to be camera specific, we recommend determining the curve that best fits the data. Although many curves will no doubt fit the data very closely (e.g. a Modified Hoerl and Weibull model, amongst others, fitted our reflection data very well), it is preferable to choose a function that is the same for each of the camera's three sensors; this makes producing the calibrations much easier because the calibration equation will be of the same form for each channel, with only the parameters varying. If there are several curves that all fit the data well, then choosing the simplest equation and with the lowest number of parameters makes calibration much easier. The equation of the curve to produce a calibration should

be reversible, favouring a simpler model, because trying to revert a high-order polynomial, for example, can be very complicated. We found that the function below fitted our camera well:

$$Q_s = a \times b^P \quad (1)$$

where  $Q_s$  is the photon catch of a given sensor  $S$  (R, G, or B),  $P$  the value of the pixel of sensor  $S$ , and  $a$  and  $b$  are constants.  $Q_s$  is the product of the measured radiance spectrum and the sensor's spectral sensitivity, but it is rare for manufacturers to publish such data. Westland & Ripamonti (2004) mention that luminance is sometimes used as an approximation, on the assumption that for a grey standard the radiance in all three channels should be the same. However, this assumes a spectrally flat light source, which no light source ever really is. Therefore, the spectral sensitivity needs to be measured directly, by measuring the camera RGB values when imaging greyscales illuminated through narrow band-pass filters. In this way, one can construct spectral sensitivity curves analogous to the spectral sensitivity curves of animal photoreceptors (Fig. 1). Párraga (2003) provides technical details on how to achieve this.

In Párraga's (2003) linearization exercise, the value of  $b$  in the equation above was found to be similar for all conditions tested (sunny, cloudy, and incandescent artificial light) and all sensors. Thus, the value of  $a$  defined each curve. Because the linearized values for R, G, and B represented radiance in the wavebands specific to each sensor, the photograph's exposure was also taken into account in the calibration process (a longer exposure time representing lower radiance). Therefore, the following three equations were derived to linearize and scale each RGB value to radiance measures, where  $Q_s$  is the radiance measured by sensor  $S$ ,  $b$  and the  $a_i$  are the coefficients estimated by ordinary least-squares regression of log-transformed values,  $c$  is a value to account for inherent dark current (see below) in the camera, and  $t$  is the integration time the photograph was taken on [1/shutter speed]:

$$Q_R = a_1(b^R - c_1)/t \quad (2)$$

$$Q_G = a_2(b^G - c_2)/t \quad (3)$$

$$Q_B = a_3(b^B - c_3)/t \quad (4)$$

If the object of the research is to obtain reflection rather than radiance measures, then  $t$  can be ignored and functions such as eqn. 1 could be used, provided that  $t$  is constant and measurements of known reflection standards are also made. Because the reflection values of greyscales are, by definition, equal in all wavebands, sensor spectral sensitivity does not in principle need to be known for linearization in relation to reflection, although in practice, one would want to know the spectral sensitivity curves for the camera's

sensors for the data to be readily interpreted (in terms of the sensitivity corresponding to each sensor). In the case of either radiance or reflection calibration, one should check that it is valid to force the calibration curve through the origin. All digital imaging sensors have associated with them an inherent 'dark current' (due to thermal noise in the sensors) (Efford, 2000; Stokman, Gevers & Koenderink, 2000; Barnard & Funt, 2002; Martinez-Verdú *et al.*, 2002), so that a set of images with the lens cap on may not produce measurements of zero. As with spectrometry, the dark current can be estimated by taking images at the same exposure settings as calibration photos, and using the pixel values as an offset for the curve. One should also check whether increasing the integration time, or temperature changes within the range at which the camera will be used for data collection, alters these background dark current values.

Figure 7 provides an example of linearization performed on the RGB values from photographs of reflectance standards (Fig. 6). This shows that, generally, the linearization was successful. However, one should note that the values of the reflectance standards with low nominal reflection values are not accurate because these standards were partially underexposed (i.e. there are many pixels with values equal or close to the dark current values) and, for this specific set of images of standards, some standards are slightly closer to, or further away from, the light source. This means that the calibration line will not be perfectly straight. Because the relatively darker areas (low pixel values) of images are often inaccurate in the measurements

they yield, these values may be nonlinear (Barnard & Funt, 2002). However, the measurement error is relatively small.

### RGB EQUALIZATION

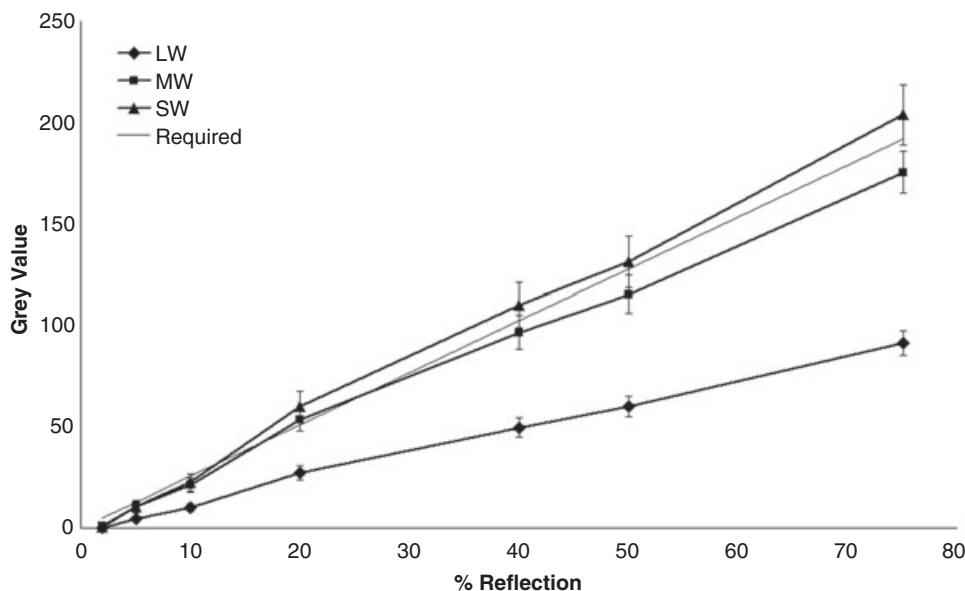
If the goal is to derive reflection data from the photographs, then greys should, by definition, have equal reflection in all three colour channels. So, if  $R \neq G \neq B$  in the calibration images, the next step is to equalize the three channels with respect to the images of the reflection standards, and then scale the values between 0–255. This, in theory, should be relatively simple: it is a matter of producing a ratio between the three channels and then scaling them, usually with respect to the green channel as a reference point, before multiplying the entire image by 2.55 to set the values on a scale between 0 and 255. So, for our data:

$$R' = (Rx_R)2.55 \quad (5)$$

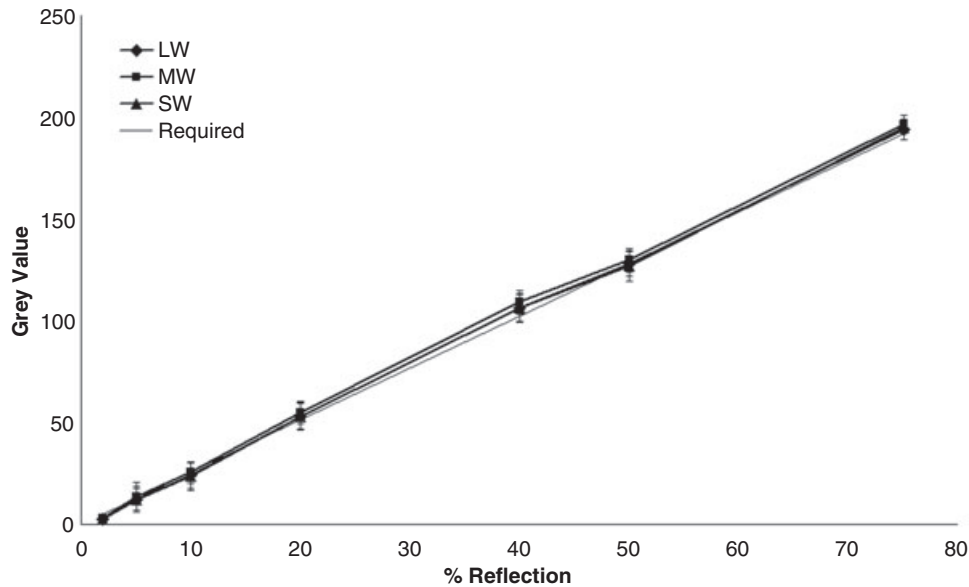
$$G' = (Gx_G)2.55 \quad (6)$$

$$B' = (Bx_B)2.55 \quad (7)$$

where  $x_i$  is the scaling value for each channel, and  $R$ ,  $G$ , and  $B$  are the linearized image values for each channel, respectively. The equalized values were then tested for accuracy using a different set of calibration images. Figure 8 shows the result. The three channels closely match the required calibration line. Note that there is no need for 255 to represent 100% reflection; indeed, to obtain maximum resolution in colour dis-



**Figure 7.** The relationship between measured greyscale value and nominal reflection value for the seven reflectance standards, showing the linearization of the gamma curves. LW, longwave; SW, shortwave; MW, mediumwave.



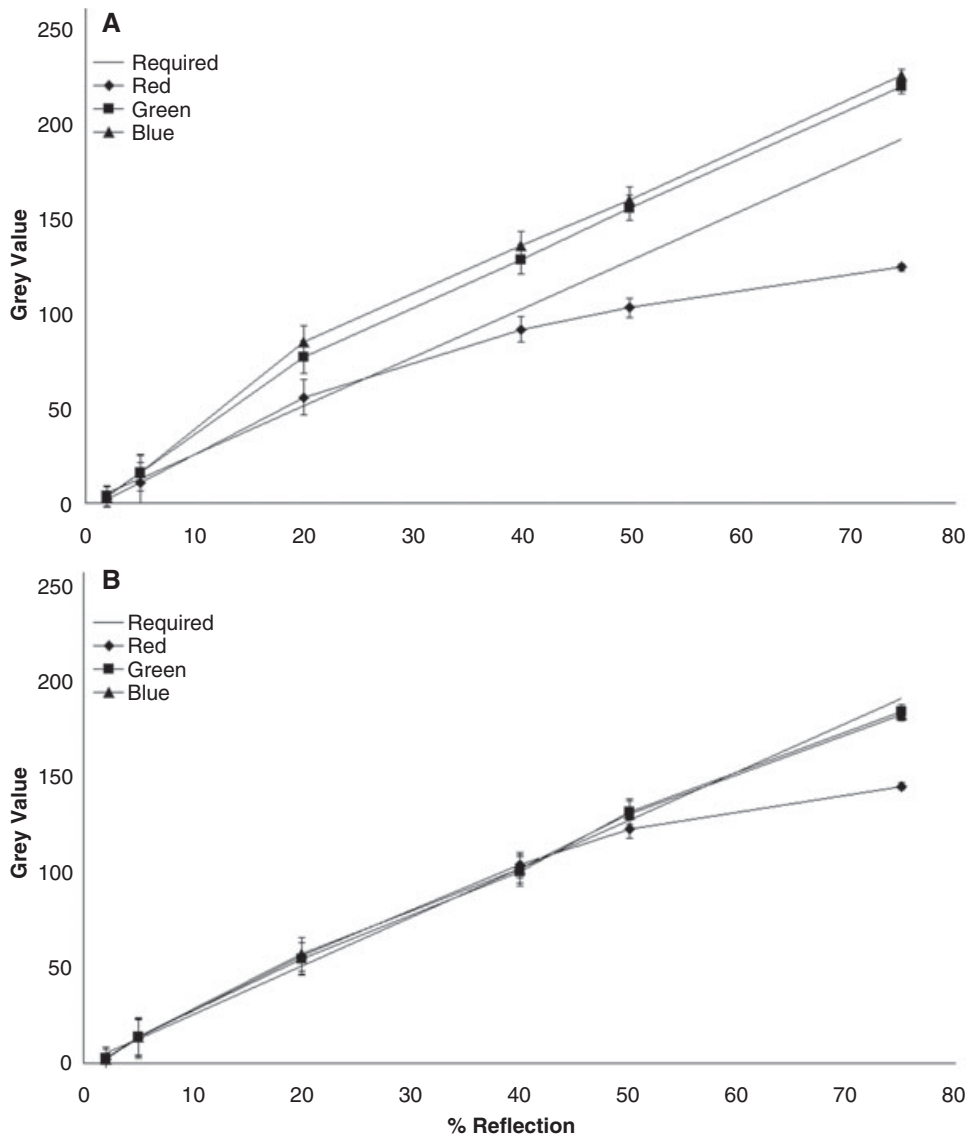
**Figure 8.** The greyscale values measured for the set of reflectance standards following the process of RGB channel equalization and scaling, showing a close fit to the required values. LW, longwave; SW, shortwave; MW, mediumwave.

crimination within and between images, if all images to be analysed are relatively dark then it would be advisable for the maximum pixel value within the dataset to be 255.

An important issue is that of saturation. With regards to the above calibration results (Figs 6, 7, 8), we maintained an integration time of 1/30 s and a lens aperture of f/8.0. This resulted in images that were slightly under-exposed and guarded against the serious problem of saturation. Saturation (also known as ‘clipping’; Lauzière *et al.*, 1999) occurs when the light levels arriving at the sensors reaches an upper limit, above which any more photons are not registered. This can be a serious problem because it prevents measurements of the true value that the pixels would have reached had saturation not occurred; a problem recognized in some studies (Hong *et al.*, 2001). The effects of saturation are easy to find, with saturated pixels in the original image yielding values of approximately 255, with little or no standard deviation. For example, images taken under similar circumstances, but with an integration time of 1/15 s produce results that at nominal reflection values of 75%, the red channel ceases to rise in pixel values. This is due to the effects of saturated pixels in the original image in the red channel, which causes the calibration to fail, since the linearization becomes ineffective and the equalization procedure results in the red channel grey values dropping away at higher reflection values (Fig. 9). These problems can be avoided by changing the exposure/integration time

(*t*), or altering the intensity of the light source, because these determine the flux of light reaching the camera’s sensors (Hong *et al.*, 2001). However, if the exposure is to be changed between images it is important to test that the response of the camera is the same at all exposure settings, otherwise a separate calibration will need to be performed for every change in exposure. Therefore, where possible, it is recommended that the aperture value, at least, is kept constant (Hong *et al.*, 2001).

It is often the case that the red channel of a digital camera is the first to saturate (as was the case with our camera, even when using a light source biased towards shorter wavelengths of light; Fig. 9), possibly because the sensors in some cameras may be biased to appeal to human perceptions, with increasing red channel values giving the perception of warmth. This may be particularly deleterious for studies investigating the content of red signals (Frischknecht, 1993; Wedekind *et al.*, 1998), which are widespread because of the abundance of carotenoid-based signals in many taxa (Grether, 2000; Pryke, Lawes & Andersson, 2001; Bourne, Breden and Allen, 2003; Blount, 2004; McGraw & Nogare, 2004; McGraw, Hill & Parker, 2005) and theories linking carotenoid signals to immune function (Koutsos *et al.*, 2003; McGraw & Ardia, 2003; Navara & Hill, 2003; Grether *et al.*, 2004; McGraw & Ardia, 2005). Some cameras are also biased in their representation of relatively short wavelengths, to compensate for a lack of these wavelengths in indoor lights (Lauzière *et al.*, 1999).



**Figure 9.** The greyscale values measured for the set of reflectance standards following the process of linearization (A) and then RGB channel equalization (B) and scaling, showing that the linearization does not produce a linear response when there are saturated pixels in the image, as is the case in the R channel in this example. Saturated pixels also result in a poor equalization result, indicated by a dropping off of the R channel at higher values.

### SELECTING/CONTROLLING LIGHT CONDITIONS

To some extent, the importance of selecting standardized lighting conditions and distances depends upon the calibration required. Lighting conditions should be as stable, standardized, and consistent as possible for each photo if measurements of reflection are desired, especially if photographs of standards are taken only at the beginning and end of sessions. However, when photographing natural scenes and using measures of photon catch, for example, lighting conditions are likely to vary considerably. This may in fact be an

important part of the study: to include information about the ambient light. Generally, it is often best to avoid flashguns because the output of these is difficult to measure and may be variable; however, a high-end flash with good light diffusers may be fine. If using a flash, putting a grey standard(s) of known reflectance into the part of the scene interested in should allow good recovery of reflectance, even if the illumination conditions vary in an uncontrolled manner, although these standards may need to be included in every image rather than just at the start/end of sessions. Therefore, using a flash may be acceptable if one is just interested in reflectance, but should be avoided if



you are interested in the behaviour of natural illumination (e.g. shadows).

### MAPPING TO CAMERA-INDEPENDENT MEASURES

Having used the coefficients obtained in the linearization procedure to linearize the RGB values in the images obtained, the next step is to transform them to camera-independent values. This is because the R, G, and B data, whether in radiance or reflectance units, are specific to the wavebands designated by the camera sensors' spectral sensitivity curves (Fig. 1C). This may be sufficient for some research purposes; for example, if the sensitivities of the camera's sensors broadly correspond to the bandwidths of interest. However, it will often be desirable, either because a specific visual system is being modelled (e.g. human, bird), or simply to facilitate comparison of the results across studies, to transform the camera-specific RGB values to camera-independent measures. In human studies, these are frequently one of the sets of three-coordinate representations devised by the CIE for colour specification and/or matching. Different three-variable representations have been devised to approximate colour-matching for images illuminating only the M-L-cone-rich central fovea, or wider areas of the retina; for presentation of images on video display units or printed paper; or representations that incorporate the colour balance arising from a specific illuminant, or are illumination independent (Wyszecki & Stiles, 1982; Mollon, 1999; Westland & Ripamonti, 2004). The advantage is that all these metrics are precisely defined, the formulae downloadable from the CIE website, and the values in one coordinate system can be transformed to another. Westland & Ripamonti (2004) provide formulae and downloadable MATLAB (The Mathworks Inc.) code for such transformations.

Another possible camera-independent transformation is to map the linearized RGB values to the spectral sensitivities of the photoreceptors of either humans (Párraga *et al.*, 2002) or nonhuman species. In the case of RGB radiance measures, this corresponds to calculating the photon catches of an animal's photoreceptors, rather than the camera's sensors, when viewing a particular scene. In the case of RGB reflectance measures, this can be thought of as a mapping to a species-specific estimate of reflectance in the wavebands to which the animal's photoreceptors are sensitive. Both types of mapping are particularly relevant to studies involving nonhuman animals, where accurate psychophysical estimates of colour-matching, of the sort used to calculate human-perceived colour from camera data, are not usually available. For such mapping to be viable, it is not necessary that the species' cone spectral sensitivities

match those of the camera's sensors particularly closely (e.g. this is not true for humans; compare Fig. 1A, C). However, for the transformation to produce reliable data, the species' overall spectral range has to fall within that of the camera, and the species has to have three or less photoreceptors. For example, one can map RGB data to the lower dimensional colour space of a dichromatic dog (with one short- and one medium/long-sensitive cone type; Jacobs, 1993), but a camera with sensitivities such as that shown in Fig. 1C can never capture the full colour world of a trichromatic bee (with UV, short-, and medium-wave photoreceptors; Chittka, 1992). Mapping RGB data to a bird's colour space would appear to be invalid on two counts: birds have a broader spectral range than a conventional camera (often extending into the UV-A) and are potentially tetrachromatic (Cuthill *et al.*, 2000b). However, if the scenes or objects of interest lack UV information, then a mapping from RGB to avian short-, medium-, and long-wave cone sensitivities can be achieved. We present the method here, which can be used for any analogous trichromatic system (e.g. human) or, with simple modification, a lower-dimensional system of the type that is typical for most mammals (Jacobs, 1993). Subsequently, we consider how UV information from a separate imaging system can be combined with the RGB data to provide a complete representation of bird-perceived colour.

The goal is to predict the quantal catches,  $Q_i$ , of a set of  $i$  photoreceptors (where  $i \leq 3$ ), given a triplet of camera-sensor-estimated radiance values,  $Q_R$ ,  $Q_G$ , and  $Q_B$ , derived from the calibration and linearization process described above. This amounts to solving a set of simultaneous regression equations, which are likely to be nonlinear. Mappings can be performed for more than three photoreceptor classes, provided that the spectral sensitivities of all types are covered by the spectral range of one or more of the camera's sensors. For example, a mapping could be produced to calculate images corresponding to the longwave, mediumwave, and shortwave cones of a bird's visual system, plus a luminance image based on avian double cone sensitivity. Once mapped images have been obtained, further calculations also allow the production of images corresponding to various opponency channels. Westland & Ripamonti (2004) summarize their, and other, research on the family of equations most likely to provide a good fit to data, and conclude that linear models (with interaction terms) of the following type perform well. For ease of interpretation, we use the notation R, G, and B to describe the camera pixel values rather than their calibrated and linearized equivalents,  $Q_R$ ,  $Q_G$ , and  $Q_B$ .

$$Q_i = b_{i1}R + b_{i2}G + b_{i3}B + b_{i4}RG + b_{i5}RB + b_{i6}GB + b_{i7}RGB \quad (8)$$

Where  $b_i$  are coefficients specific to receptor  $i$ , and the curve is forced through the origin (when the calibrated camera sensor value is zero, the animal's quantal catch is zero). In some cases, dependent on the camera and the nature of the visual system to which mapping is required, polynomials (i.e. including terms in  $R^2$ ,  $G^2$ , and  $B^2$ , or higher orders) may provide a significantly better fit (and did in our case); this should be investigated empirically. Cheung *et al.* (2004) note that even mapping functions of unconstrained form, obtained using neural networks applied to large datasets, do not significantly outperform polynomials. The data required to estimate the coefficients for the  $i$  photoreceptors can either be radiances directly measured using an imaging spectroradiometer (Párraga, 2003) or, more conveniently, radiances approximated as the product of reflectance spectra and the irradiance spectrum of the illuminant. Using eqn. 8, applied to a trichromat,  $3 \times 7$  coefficients need to be estimated, so the number of radiance spectra must be considerably greater than this ( $> 100$  in our experience as a minimum, but closer to a 1000 is better). Large numbers of radiance spectra can be obtained from internet databases (Parkkinen, Jaaskelainen and Kuittinen, 1988; Sumner & Mollon, 2000). The coefficients for each photoreceptor are then found by multiple regression (or, conveniently, if using MATLAB, by matrix algebra; Westland & Ripamonti, 2004). Although, in principle, one could derive a mapping function (i.e. set of coefficients) for all possible natural spectra, viewed under all possible illuminants, greater precision can be achieved by determining a situation-specific mapping function for the research question at hand. For example, if the goal is to use a camera to quantify the coloration of orange to red objects under blue skies, then a very precise mapping function could be estimated by using radiance data calculated only from the reflectance spectra of orange to red objects viewed under blue sky irradiance. If one is to derive the mapping functions by calculation (i.e. calculate quantal catch for camera and desired cone sensitivities, using reflectance and irradiance data), then the sensitivity of the camera's sensors is required. However, one could also derive the mapping empirically without ever measuring camera sensor sensitivities, by measuring the response of the camera's three channels to different (known) radiance spectra, and by determining the response of the cones of the required animal's visual system. To achieve accurate mapping, the camera's response would have to be measured for many hundreds of radiance spectra and this would be time-consuming, involving many stimuli.

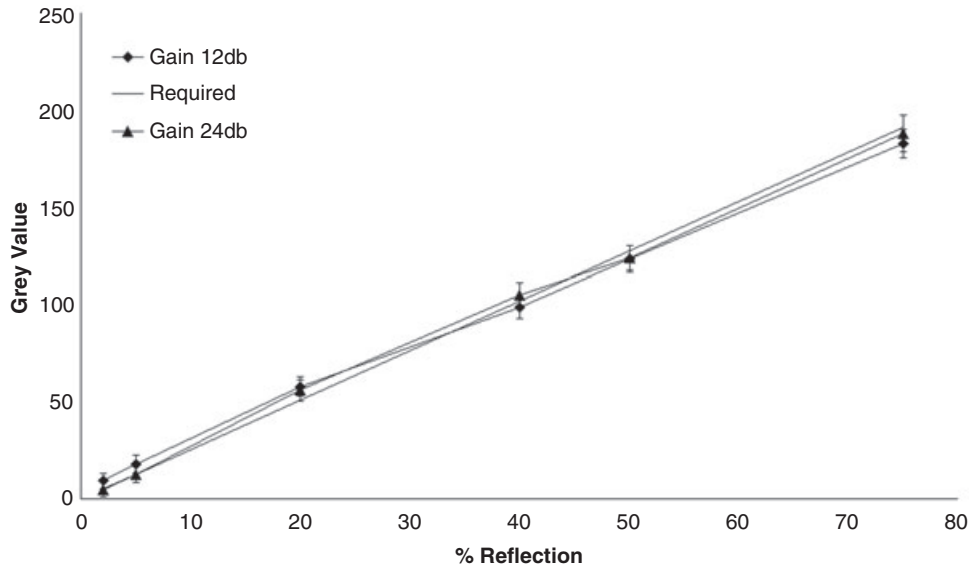
## UV IMAGING

In our own research, we wished to quantify lepidopteran wing patterns, with respect to avian vision,

so we also needed to measure the amount of reflection in the avian-visible UV waveband. At the same time as RGB photography, images of the reflectance standards and the lepidopterans were taken with a UV sensitive video camera (see Appendix 2).

First, we tested whether the camera was linear with respect to both changes in the integration time, and with respect to increases in the reflection value; being a high-specification technical camera, this was indeed the case. This meant that the only calibrations needed were to scale the images to between 0 and 255; which is not initially as easy as it sounds because the calibrations have to account for different gain and the integration times. Figure 10 provides an example of the results for the UV calibration process. In most situations, it will be simpler to maintain the same gain values because this reduces the number of factors to consider in the calibration process.

If images are obtained from more than one camera, there is an additional consideration that must be addressed; that of 'image registration'. Images derived from one RGB camera will all be the same angle and distance from the specimens, and so the objects photographed will be on an identical scale in each of the three channels, based on the interpolations implemented. This may not be the case if obtaining images from a second camera; such as in our study, meaning that the specimens were a different size in the photographs and would not necessarily be easy to align with the RGB images. Furthermore, one camera may produce images with a lower resolution, and with less high frequency information; different cameras will have different Nyquist frequencies, meaning that although aligning lower spatial frequency patterns may be relatively easy, information may be lost or poorly aligned at higher frequencies. One potential approach is to use Fourier filtering to remove the highest spatial frequency information from those images that contain it, down to the highest frequencies contained in the images from the other camera. However, this may be undesirable if the high spatial frequency information is important, as it frequently will be with complex patterns, or where edge information between pattern components is critical. The task of aligning images is made easier if: (1) different cameras are set up as closely as possible, in particular with relation to the angle of photography because this is the hardest factor to correct and (2) rulers are included in at least a sample of the images, so they can be rescaled to ensure specimens occupy the same scale in different images. Including rulers in images allows for true distance measurements to be obtained and for spatial investigations to be undertaken. If images from one camera are larger than those from another, then it is the larger images that should be scaled down in size because this avoids artefactual data, generated by



**Figure 10.** The effect of scaling the ultraviolet (UV) images obtained with the PCO Variocam camera and Nikon UV transmitting lens, showing a close fit to the required values.

interpolation, if images are rescaled upwards. Once the objects in the photographs are of the same size, it may be a relatively trivial task to take measurements from the different images that directly correspond. However, if the images are still difficult to align then an automated computational approach can be used. A variety of these are available, and users should carefully consult available manuals/information for the corresponding software to be sure of how the registration is completed, and to check what changes may occur to the image properties. However, in many cases, changes to the image structure will probably be small, especially at lower spatial frequencies, and have little influence on the results. One such plug-in, for the free-ware software Image J (Rasband, 1997–2006; Abramoff *et al.*, 2004), is ‘TurboReg’ (available via a link from the Image J website) (Thévenaz, Ruttimann & Unser, 1998), which comes with a variety of options to align sets of images.

#### HOW BEST TO USE COLOUR STANDARDS

A crucial step in calibrating a digital camera is to include colour standards in some or all of the photographs taken. Including a set of colour standards in each photo allows calibrations to be derived for each individual photo, which would be highly accurate. However, in most cases, this is impractical and unnecessary. For example, when the light source used is consistent, a set of reflectance standards used to fit a calibration curve need only be included in photos at the start and end of a session. Including these in each photo may leave little space for the objects of interest.

By contrast, in many cases, such as when photographing natural scenes where the illuminating light may change and when wishing to calculate values such as photon catches, it may be important to include at least one grey standard in the corner of each photo. Possibly the best objects to include in a photo are Spectralon reflectance standards (Labsphere Inc.), which reflect a known amount of light equally at all wavelengths in the UV and human visible spectrum. However, these are expensive and easily damaged, and if a single standard is sufficient, a Kodak grey card (Eastman Kodak Company), which has an 18% reflectance, can be included, which is relatively inexpensive.

#### SPATIAL MEASUREMENTS

Often, we do not wish to measure solely the ‘colour’ of a patch, but the area or shape of a region of interest. In principle, this sounds easy but has several complications. For example, the colour boundary of an area visible to humans may not be exactly the same as for that of another animal. Additionally, there may be colours that we cannot see (such as UV) that have different boundaries to those visible by a human (although most colour patches generally have the same boundary for different colour bands, such as UV, SW, MW, and LW). Another problem corresponds to the acuity of the animal in question. Regions of interest with complex boundaries may be only discernable by animals with a high enough spatial acuity. Furthermore, there is a specific problem with gradual boundaries, particularly relating to defining where the actual edge of the colour region is.

There are several ways to address these issues and one must remember that the image processing steps that facilitate patch size or shape measurement may interfere with the accurate measurement of patch colour *per se* (e.g. by enhancing contrast between patches). One method of determining the boundary of a colour patch is to produce an automated procedure to define a specific area of interest. This can be done by thresholding an 8-bit or colour image to a binary (black and white) image where each individual pixel has a value of either one (white) or zero (black) (Fig. 11). This can be performed by writing a custom programme where the threshold level is defined specifically by the user, preferably based on an explicit assumption or data. Otherwise, most imaging software has automatic thresholding algorithms, although it is not always known what the thresholding value used will be.

A different method that can be used to define an area of interest is that of edge detection. This is where an algorithm is used to determine edges in an image, corresponding to sharp changes in intensity (either luminance or in terms of individual colour channels). These edges may, for example, be found at the boundary of a colour patch (Fig. 11). The useful thing about edge detection algorithms is that they can be optimized and not linked to any specific visual system, or they correspond to the way in which real visual systems work (Marr & Hildreth, 1980; Bruce, Green & Georgeson, 2003; Stevens & Cuthill, 2006).

Once the boundary of a colour patch has been defined, it is simple to measure the area of the patch. Measuring the shape of an object is more difficult, although imaging software often comes with algorithms to measure attributes such as the relative circularity of an area and, occasionally, more advanced shape analysis algorithms.

#### DRAWBACKS TO USING DIGITAL IMAGES

The most notable drawback is that the information obtained is not wavelength specific (i.e. it is known what wavelengths contribute to each channel, but not the contribution of any specific wavelength to the RGB value of any one pixel). This drawback can be overcome by so-called multispectral imaging (or, if the number of wavebands is high, 'hyperspectral imaging'). This can involve rotating a set of filters in front of the lens, allowing the acquisition of successive images of different wavebands (Brelstaff *et al.*, 1995; Lauzière *et al.*, 1999; Angelopoulou, 2000; Stokman *et al.*, 2000; Losey, 2003). This method may be particularly useful if detailed wavelength information is required, or if the visual system of the receiver that the signal is aimed at is poorly matched by the sensitivity of an RGB camera. We do not cover this tech-

nique here because, although it combines many of the advantages of spectrometry with photography, the technology is not practical for most behavioural and evolutionary biologists. Hyperspectral cameras are often slow because they may have to take upwards of 20 images through the specified spectral range. The equipment, and controlling software, must be constructed *de novo* and conventional photography's advantage of rapid, one-shot, image acquisition is lost. The specimens must be stationary during the procedure because movement can cause problems with image registration. Also, as Losey (2003) acknowledges, images obtained sequentially in the field may be subject to short-term variations in environmental conditions, and thus introduce considerable noise. Averaging the values obtained from multiple frames of the same waveband may help to eliminate some of this effect (Losey, 2003).

#### PROBLEMS WITH USING THE AUTOMATIC CAMERA SETTINGS

Many studies of animal coloration utilizing cameras apparently use the camera with its automatic settings. There are numerous problems that can arise when using the 'auto' mode. The main problem is that the settings used by the camera are adjusted according to the scene being photographed and so may be inconsistent. In general, camera manufacturers are interested solely in selling cameras and therefore want to produce pictures that look aesthetically 'good' by enhancing some of the images' colours and contrasts and, thus, automatic modes are generally compatible with this objective. Given that an automatically set white balance changes between photos, it gives rise to different ratios between the LW, MW, and SW sensor responses. This need not always be an irretrievable flaw but would almost certainly need some highly complex calibration procedures to recover consistent data, such as calibrating every single combination of white balance, contrast enhancement and aperture setting modes. Any low- to mid-range camera is likely to have some white balancing present, and most mid-range cameras will give the option to manually set the white balance. If the camera does not allow this option and there is no indication of this in the manual, then changing the white-balance settings may not be possible. An additional problem with automatic settings is that calibration curves/settings could also change at different aperture settings; this may not always be the case but, when using the automatic mode, there is there additional complication that the aperture and exposure (integration) time may change significantly simultaneously, leading to unnecessarily complicated calibrations if values of reflection, for example, are required. The aperture selected by the



**Figure 11.** Different images of a clouded yellow butterfly *Colias croceus*, modified to show regions of interest, such as the wing spots, identified by various techniques. A, the original 8-bit grey-level image (pixel values between 0 and 255). B, the image after an edge detection algorithm has been applied, clearly identifying a boundary around the two forewing spots, but not the hindwing spots. C, the original image after being thresholded to a binary (black/white) image with a threshold of 64. This clearly shows the forewing spots but does not produce spots where the hindwing spots were in the original image. D, the original image when converted to a binary image with a threshold of 128, now picking out both the forewing and hindwing spots (although with some 'noise' around the hindwing spots). E, the original image converted to a binary image with a threshold of 192, now not showing any clear wing spots. F, the original image when first converted to a pseudocolour image, where each pixel value falling between a given range is given a specific colour. The image is then reconverted to a grey-level image and now shows the hindwing spots with marginally sharper edges than in the original image.

camera will also affect the quality of the image, particularly depth of field. Another potentially serious problem with using the auto mode is that the photograph will not optimize the dynamic range of the scene photographed, meaning that some parts of the scene may be underexposed or, far more seriously, saturated.

## CONCLUSIONS

One of the earliest studies to outline how digital image analysis can be used to study colour patterns is that of Windig (1991), with an investigation of lepidopteran wing patterns. Windig (1991) used a video camera, connected to a frame grabber to digitize the images for computer analysis, a similar method to that which we used to capture the UV sensitive images. Windig (1991) stated that the method was expensive, and the programmes were highly complex but, today, flexible user friendly software is available, with various free-ware programmes downloadable off the internet, and the purchase of a digital camera and software is possible for a fraction of the cost of setup used by Windig (1991).

Windig (1991) argued that any image analysis procedure should meet three criteria. First, completeness: a trait should be quantified with respect to all characters, such as 'colour' and area. Our procedure meets this criterion because reflection, plus spatial measurements, are attainable. Second, the procedure needs to be repeatable. This was also the case with our approach because the calibrations for a set of images of reflectance standards were still highly accurate for other images taken under the same conditions, but at different times. Finally, the process should be fast relative to other available methods, as was our study, with potentially hundreds of images taken in a day, quickly calibrated with a custom MATLAB programme and then analysed with the range of tools available in Image J.

Another advantage of capturing images with a digital camera is that there are potentially a host of other noncolour analyses. Detailed and complex measurements of traits can be undertaken rapidly, with measurements and calculations that would normally be painstakingly undertaken by hand performed almost instantaneously in imaging software, including measurements of distances, areas, and analysis of shapes, plus complex investigations such as Fourier analysis (Windig, 1991). This may be particularly useful if handling the specimens to take physical measurements is not possible.

The use of digital technology in studying animal coloration is a potentially highly powerful method, avoiding some of the drawbacks of other techniques. In future years, advances in technology, software, and our understanding of how digital cameras work will

add further advantages. It is already possible to extract data of a scene from behind a plane of glass (Levin & Weiss, 2004), which could become useful for studies of aquatic organisms (although most glass filters out UV wavelengths; Lauzière *et al.*, 1999). Techniques are also being developed to remove the shadows from images; shadows can make edge recognition more difficult (Finlayson, Hordley & Drew, 2002), and hinder tasks such as image registration. With the explosion in the market of digital photography products, and the relatively low cost to purchase such items, there is the temptation to launch into using such techniques to study animal signals, without prior investigation into the technicalities of using such methods. This could result in misleading results. Therefore, although digital photography has the potential to transform studies of coloration, caution should be implemented and suitable calibrations developed before such investigations are undertaken.

## KEY POINTS/SUMMARY

Below is a list of some of the main points to consider if using cameras to study animal coloration.

1. Images used in an analysis of colour should be either RAW or TIFF files and not JPEGs.
2. Grey reflectance standards should be included in images at the start of a photography session if the light source is constant, or in each image if the ambient light changes.
3. It is crucial not to allow images to become saturated or underexposed because this prevents accurate data being obtained.
4. Many cameras have a nonlinear response to changes in light intensity, which needs linearizing before usable data can be obtained.
5. To produce measurements of reflectance, the response of the R, G, and B colour channels needs to be equalized with respect to grey reflectance standards.
6. Measurements of cone photon catches corresponding to a specific visual system can be estimated by mapping techniques based upon sets of radiance spectra and camera/animal spectral sensitivity.
7. Digital images can be incorporated into powerful models of animal vision.
8. Do not convert image data to formats such as HSB, which are human-specific and inaccurate. Instead, use reflection data, calculations of photoreceptor photon catches or, if working on human-perceived colour, well-tested colour spaces such as CIE.
9. If using more than one camera, image registration may be a problem, especially if the different cameras have different resolutions. This problem can be minimized by setting up different cameras as

close to one another as possible and ensuring that one camera does not capture significantly higher levels of spatial detail than the other.

10. Digital imaging is also a potentially highly accurate and powerful technology to study spatial patterns.

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## APPENDIX 1

### GLOSSARY OF TECHNICAL TERMS

#### *Aliasing*

When different continuous signals become indistinguishable as a result of digital sampling. Spatial alias-

ing is manifested as the jagged appearance of lines and shapes in an image.

#### *Aperture*

Aperture refers to the diaphragm opening inside a photographic lens. The size of the opening regulates the amount of light passing through onto the colour filter array. Aperture size is usually referred to in f-numbers. Aperture also affects the 'depth of field' of an image.

#### *Bit depth*

This relates to image quality. A bit is the smallest unit of data, such as 1 or 0. A 2-bit image can have  $2^2 = 4$  grey levels (black, low grey, high grey and white). An 8-bit image can have  $2^8 = 256$  grey levels, ranging from 0 to 255. Colour images are often referred to as 24-bit images because they can store up to 8 bits in each of the three colour channels and therefore allow for  $256 \times 256 \times 256 = 16.7$  million colours.

#### *Charge-coupled device (CCD)*

A small photoelectronic imaging device containing numerous individual light-sensitive picture elements (pixels). Each pixel is capable of storing electronic charges created by the absorption of light and producing varying amounts of charge in response to the amount of light they receive. This charge converts light into electrons, which pass through an analogue-to-digital converter, which produces a file of encoded digital information.

#### *Chromatic aberration*

This is caused by light rays of different wavelengths coming to focus at different distances from the lens causing blurred images. Blue will focus at the shortest distance and red at the greatest distance.

#### *Colour filter array*

Each pixel on a digital camera sensor contains a light sensitive photodiode which measures the brightness of light. These are covered with a pattern of colour filters, a colour filter array, to filter out different wavebands of light.

#### *Demosaicing algorithms*

Most digital cameras sample an image with red, green, and blue sensors arranged in an array, with one type at each location. However, an image is required with an R, G, and B-value at each pixel location. This is produced by interpolating the missing sensor values via so called 'demosaicing' algorithms, which come in many types.

#### *Exposure*

The exposure is the amount of light received by the camera's sensors and is determined by the aperture and the integration time.

*Foveon sensors*

Foveon sensors capture colour by using three layers of photosensors at each location. This means that no interpolation is required to obtain values of R, G, and B at each pixel.

*Image resolution*

The resolution of a digital image is the number of pixels it contains. A 5-megapixel image is typically 2560 pixels wide and 1920 pixels high and has a resolution of 4915 200 pixels.

*JPEG*

JPEG (Joint Photographic Experts Group) is very common due to its small size and widespread compatibility. JPEG is a lossy compression method, designed to save storage space. The JPEG algorithm divides the image into squares, which can be seen on badly compressed JPEGs. Then, a discrete cosine transformation is used to turn the square data into a set of curves, and throws away the less significant part of the data. The image information is rearranged into colour and detail information, compressing colour more than detail because changes in detail are easier to detect. It also sorts detail information into fine and coarse detail, discarding fine detail first.

*Lossy compression*

A data compression technique in which some data is lost. Lossy compression attempts to eliminate redundant or unnecessary information and dramatically reduces the size of a file by up to 90%. Lossy compression can generate artefacts such as false colours and blockiness. JPEG is an image format that is based on lossy compression.

*Lossless compression*

Lossless compression is similar to 'zipping' a file, whereby if a file is compressed and later extracted, the content will be identical. No information is lost in the process. TIFF images can be compressed in a lossless way.

*Macro lens*

A lens that provides continuous focusing from infinity to extreme close-ups.

*Modulation transfer function*

The modulation transfer function describes how much a piece of optical equipment, such as a lens, blurs the image of an object. Widely spaced features, such as broad black and white stripes, do not lose much contrast, because a little blurring only affects their edges, but fine stripes may appear to be a uniform grey after being blurred by the optical apparatus. The modula-

tion transfer function is a measure of how much bright-to-dark contrast is lost, as a function of the width of the stripes, as the light goes through the optics.

*Nyquist frequency*

The Nyquist frequency is the highest spatial frequency where the CCD can still correctly record image detail without aliasing.

*RAW*

A RAW file contains the original image information as it comes off the sensor before internal camera processing. This data is typically 12 bits per pixel. The camera's internal image processing software or computer software can interpolate the raw data to produce images with three colour channels (such as a TIFF image). RAW data is not modified by algorithms such as sharpening. RAW formats differ between camera manufacturers, and so specific software provided by the manufacturer, or self written software, has to be used to read them.

*Saturation*

In the context of calibrating a digital camera, we use this term to denote when a sensor reaches an upper limit of light captured and can no longer respond to additional light. This is also called 'clipping' as the image value cannot go above 255 (in an 8-bit image) regardless of how much additional light reaches the sensor. Saturation can also be used to refer to the apparent amount of hue in a colour, with saturated colours looking more vivid.

*Sensor resolution*

The number of effective non-interpolated pixels on a sensor. This is generally much lower than the image resolution because this is before interpolation has occurred.

*TIFF*

TIFF (Tagged Image File Format) is a very flexible file format. TIFFs can be uncompressed, lossless compressed, or can be lossy compressed. While JPEG images only support 8 bits per channel RGB images, TIFF also supports 16 bits per channel and multilayer CMYK images in PC and Macintosh format.

*White balance*

Most digital cameras have an automatic white balance setting whereby the camera automatically samples the brightest part of the image to represent white. However, this automatic method is often inaccurate and is undesirable in many situations. Most digital cameras also allow white balance to be chosen manually.

## APPENDIX 2

## TECHNICAL DETAILS

In the present study, we used a Nikon Coolpix 5700 camera, with an effective pixel count of just under 5.0 megapixels. This does not have all of the desired features described in our paper (the intensity response is nonlinear and the zoom cannot be precisely fixed) and we offer no specific recommendation, but it is a good mid-priced product with high quality optics and full control over metering and exposure. UV photography was with a PCO Variocam, fitted with a Nikon UV-Nikkor 105 mm lens, a Nikon FF52 UV pass filter and an Oriel 59875 'heat' filter (the CCD is sensitive to near-infra-red). The camera was connected to a Toshiba Satellite 100 cs laptop and also to an Ikegami PM-931 REV.A monitor, which displayed the images that were to be saved via a PCO CRS MS-DOS based programme. With the camera remote control, the gain and the integration time of the images could be adjusted, with the gain either set to 12 db or 24 db and

the integration time between one and 128 video frames (1 frame = 40 ms).

Images were transferred to a PC and all measurements were taken with the (free) imaging programme 'Image J' (Rasband, 1997–2006; Abràmoff *et al.*, 2004). Measurements of standards were taken by drawing a box over the area of interest, and then using the histogram function to determine the mean grey scale value and standard deviation for each channel. All other image and data manipulations, including the linearization and transformation between coordinate systems, were performed with MATLAB (The Mathworks Inc.), although other languages, such as Java (Sun Microsystems, Inc.; Efford, 2000) are also useful. MATLAB has rapidly become an industry standard in vision science, on account of its efficiency at matrix mathematics and manipulation (photographic data are large matrices). MATLAB and Image J benefit from the large number of plug-ins and toolboxes written by users for other users.