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Achromatic Cues Are Important for Flower Visibility to Hawkmoths and Other Insects

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Studies on animal colour vision typically focus on the chromatic aspect of colour, which is related to the spectral distribution, and disregard the achromatic aspect, which is related to the intensity (“brightness”) of a stimulus. Although the chromatic component of vision is often most reliable for object recognition because it is fairly context independent, the achromatic component may provide a reliable signal under specific conditions, for example at night when light intensity is low. Here we make a case for the importance of achromatic cues in plant-pollinator signalling, based on experimental data on naïve *Deilephila elpenor* and *Macroglossum stellatarum* hawkmoths, optical modelling and synthesising published experiments on bees, flies, butterflies and moths. Our experiments show that in ecologically relevant light levels hawkmoths express a strong preference for brighter stimuli. Published experiments suggest that for flower-visiting bees, butterflies, moths and flies, achromatic cues may be more important for object detection than often considered. Our optical modelling enabled disentangling the contribution of pigments and scattering structures to the flower’s achromatic contrast, and illustrates how flower anatomy and background are important mediating factors. We discuss our findings in the context of the often-assumed dichotomy between detection and discrimination, chromatic versus achromatic vision, and the evolution of floral visual signals.

Keywords: brightness, hawkmoth, insect colour vision, pollination, achromatic contrast, achromatic vision, flower colour, pigment

INTRODUCTION

Many animals rely on colour information for numerous tasks, such as finding mates and food or avoiding predators (reviewed by Kelber et al., 2003; Cronin et al., 2014; van der Kooi et al., 2021). Colour has a chromatic aspect, which is related to the spectral distribution of the reflected or emitted light, and an achromatic aspect, which is related to the intensity or total reflectance of a stimulus (Kelber et al., 2003; Osorio and Vorobyev, 2005; Kemp et al., 2015). Hue and saturation are terms used to characterise human perception of the chromatic aspect, and brightness is used to describe the achromatic aspect of colour, though these terms are not defined for animals. In this text, we use the word “bright” to describe a colour of higher intensity and “dark” for a colour of low intensity. Likewise, we will use human colour terms to describe how colours appear to the human eye.

Ever since the experiments on honeybee colour vision by von Frisch (1914), the use of chromatic information by animals has gained far more attention than their use of the achromatic aspect of colour. The use of the achromatic aspect of colour is seen as more “primitive” than the use of the chromatic aspect, because achromatic cues can be detected by a single spectral type of photoreceptor or a summed receptor signal (Osorio and Vorobyev, 2005), whereas chromatic vision depends on comparing signals from two or more spectral types of receptors (Kelber et al., 2003). Moreover, to demonstrate the use of colour vision in an animal, it is required to show that it uses chromatic information and to exclude the possibility that it used achromatic information (Kelber et al., 2003). This is usually done by making achromatic information unreliable in the experiment, which has led to the present situation that less is known about the use of achromatic than chromatic information in many animals.

In this essay, we call for an increased focus on the achromatic aspect of flower colour and make the case that achromatic information may be more important for flower detection and discrimination by insect pollinators than often acknowledged. The proposition is relevant for a better understanding of pollinator vision as well as flower colours. We will discuss this using five questions:

- (1) Do flower-visiting insects use chromatic and achromatic signals for separate purposes as often suggested?
- (2) Is there a difference between diurnal and nocturnal insects in the use of achromatic versus chromatic cues?
- (3) Is there a difference in how chromatic and achromatic cues guide the innate preferences of naïve flower visitors and learned preferences for flower colours?
- (4) Do chromatic and achromatic cues have different roles for the detection of flowers against the background, and for the discrimination between flowers?
- (5) How do floral scattering and pigmentation properties determine the achromatic contrast?

In the following, after giving a short historical background, we will summarise basics on chromatic and achromatic vision in insects. We test the importance of achromatic contrast in an experiment with diurnal and nocturnal hawkmoths, and present evidence from literature on the importance of achromatic cues in plant-pollinator signalling. We use an optical model that enables disentangling the contribution of pigments, scattering structures and the type of background to discuss what determines the achromatic contrast between flowers and their background.

HISTORICAL BACKGROUND

Why is the use of achromatic cues often neglected? First, for many years, the goal was to understand colour vision. While already in the 17-hundreds, Sprengel (1793) noted that flowers were not colourful to appeal to humans but to attract pollinators, it took until the seminal work of Turner (1910) and von Frisch (1914) that it became broadly accepted that insects use colour information. At that time, colour vision, which is based on multiple photoreceptor types and neural

comparison of their signals, was considered superior to or more derived than achromatic vision, which is possible with any spatially resolving eye.

The important notion made by von Frisch was that in order to show that an animal used colour, it needed to be clear that the animal used the chromatic aspect of colour. The achromatic aspect thus had to be negated by the experimenter (von Frisch, 1914; Kelber et al., 2003). Over the last century, colour vision has been documented in many insects, leading to the conclusion that most species use it. For example, in insects alone, the spectral sensitivities have been described in more than 200 species of 13 orders (van der Kooi et al., 2021). Behavioural studies aiming to test whether an insect species possesses colour vision need to exclude the use of achromatic information (Kelber et al., 2003; Spaethe et al., 2014; Arikawa et al., 2021), but the opposite, that is, rigorous exclusion of chromatic cues to investigate the use of achromatic cues, has very rarely been undertaken.

Second, starting with Turner’s and von Frisch’s work, there has long been a bias toward research on a single – albeit important – species of insect pollinators, European honeybees (*Apis mellifera*), because these are easy to obtain and maintain, and convenient in behavioural studies. Although research on honeybees has provided a leap in comprehension on colour vision, literature is taxonomically biased toward this species. A caveat pertaining honeybees as model system in vision research is that in honeybees there may be a comparatively strong difference between the use of chromatic and achromatic cues. Behavioural experiments suggested that honeybees (*A. mellifera* and *A. cerana*) use achromatic or chromatic information at different stages of their approach to flowers, depending on the subtended visual angle of a stimulus (e.g., Lehrer and Bischof, 1995; Giurfa et al., 1996, 1997; Menzel et al., 1997; Meena et al., 2021). Thus, in behavioural terms, honeybees use achromatic or chromatic contrast depending on the size of and distance to a flower. In other insects, this separation may not strictly be the case (e.g., in butterflies, Stewart et al., 2015).

Third, it was long assumed that insect chromatic and achromatic vision are processed by two different channels, with separate neural pathways (Table 1; for discussions, see Kelber and Henze, 2013). In the honeybee, behavioural experiments have shown that only the green photoreceptor (often called long-wavelength-sensitive receptor) is used for achromatic tasks such as motion and shape detection (Srinivasan, 1985; Srinivasan and Lehrer, 1988; Hempel de Ibarra and Giurfa, 2003). Achromatic vision is also used to control landing, a behaviour studied extensively in flies (e.g., Tinbergen and Abeln, 1983; Van Breugel and Dickinson, 2012) and bees (e.g., Lehrer et al., 1990). Patterns are very poorly detected by the bee’s achromatic channel if they only present contrast in the UV (short-wavelength-sensitive) or blue (medium-wavelength-sensitive) photoreceptor. In flies, a similar case was posited. Six morphologically similar photoreceptors (named R1-6) with the same broadband sensitivity were assumed to feed into the motion and form vision channels, whereas the remaining two receptor types (R7/8) were assumed to feed into the colour vision channel. The fly eye is a mosaic of two ommatidial types, all with the same R1-6, but with two sub-types and different spectral

TABLE 1 | Examples of insect pollinators and their chromatic and achromatic visual channels.

	N spectral types of receptors	Chromatic vision based on	Achromatic vision based on	Selected references
Apidae (Hymenoptera)	UV, Blue, and Green	UV, Blue Green	Green	Peitsch et al., 1992; Giurfa et al., 1997
Diptera	R1-6 broadband R7/8 UV1, UV2, Blue, and Green	R7/8, plus some influence of R1-6	R1-6, plus some influence from R7/8	Wardill et al., 2012; Schnaitmann et al., 2013
<i>Sphingidae</i> (Lepidoptera)	UV, Blue, and Green	UV, Blue Green	Assumed the green receptor, but not confirmed	Telles et al., 2014
<i>Papilio aegaeus</i> (Lepidoptera)	Five peak sensitivities plus broadband	UV, Blue, Green, and Red	Unknown	Arikawa, 2003
<i>Pygopleurus israelitus</i> (Coleoptera)	UV, Green, and Red	UV, Green, and Red	Unknown	Martínez-Harms et al., 2012

R indicates numbered receptor types, see references for clarification.

sensitivities in R7 (UV1 and UV2) and R8 (blue and green). Fly eyes thus have four spectrally different types of photoreceptors that are used for colour vision (Hardie, 1986). This general scheme seems to be universal among Diptera, at least for common flower visitors such as Syrphids (reviewed by van der Kooi et al., 2021).

Early work on bee and fly chromatic and achromatic colour processing led to the general assumption that achromatic and chromatic vision might be physically and behaviourally separated in insect vision in general, even though not much was known about other groups. For most insect groups, it is not clear which photoreceptor(s) is/are important for processing of achromatic information (see **Table 1**), but the green receptor is a suitable and likely candidate for the achromatic channel, because it is the most abundant photoreceptor type across insect eyes and thus, the green channel has the highest sensitivity (Kelber et al., 2003).

Over the last decade, however, work has revealed that the dichotomy in neural chromatic versus achromatic pathways is not universal, and that the pathways intersect in both flies and butterflies, meaning that chromatic processes feed into the achromatic channel and vice versa (Wardill et al., 2012; Kelber and Henze, 2013; Schnaitmann et al., 2013; Rusanen et al., 2018; Pagni et al., 2021). The same may apply to other insects such as moths and bees.

These three historical facets of studies on insect colour vision have contributed to our lack of understanding of the use of achromatic cues by flower visitors.

WHAT MAY ACHROMATIC CUES BE USED FOR?

The reliability of achromatic versus chromatic cues depends on biotic and abiotic conditions. Chromatic vision is often considered more reliable than achromatic vision, because it is less context dependent (Osorio and Vorobyev, 2005; Johnsen et al., 2006). Achromatic cues vary with illumination, for example caused by shadows or clouds, but also the spectral composition of the illumination, which differs between an open field and the forest, and between twilight, starlight and moonlight (Johnsen et al., 2006). By contrast, colour vision systems universally correct, at least partly, for illumination

differences by means of colour constancy (reviewed by Chittka et al., 2014), so enabling the animal to recognise the same colour under varying illumination.

As compared to chromatic cues, achromatic cues represent the highest signal power and information content in any image (Osorio and Vorobyev, 2005; Vasas et al., 2017). The higher signal to noise ratio of achromatic cues has been taken as main reason why motion vision, polarisation vision as well as other visual domains that require high spatial and temporal resolution of insect vision mainly rely on achromatic input (Osorio and Vorobyev, 2005). Therefore, achromatic cues could be more important for flower detection and discrimination than often assumed.

Insect responses to achromatic cues vary markedly between species. Honeybees can detect flowers from the threefold distance using achromatic contrast, compared to chromatic contrast (**Table 2**). Spaethe et al. (2001) showed that in bumblebees, the relative importance of achromatic versus chromatic cues also varies with size of, or distance to, the stimulus. Smaller flowers grouped in inflorescences can be detected from further away, if they provide green receptor contrast (Wertlen et al., 2008). However, the difference between achromatic and chromatic spatial resolution is smaller in, for example, stingless bees (Dyer et al., 2016; Jezeera et al., 2021). *Eristalis tenax* hoverflies exhibit preferences for bright (yellow) stimuli (An et al., 2018) and *Papilio xuthus* butterflies prefer high achromatic contrast between flowers and their background (Kinoshita et al., 2012), but *Catopsilia pomona*, a diurnal pierid butterfly, did not prefer yellow flower models of higher intensity (Balamurali et al., 2020).

Even animals of the same species can give different weight to chromatic and achromatic cues, depending on context. Crepuscular flower-visitors such as the hawkmoth *Manduca sexta*, albeit showing a clear chromatic preference for blue flowers in twilight (Goyret et al., 2008), switch to an achromatic preference for bright flowers in dim starlight and, if flowers are seen in front of a dark background, also in moonlight levels (Kuenzinger et al., 2019). The nocturnal hawkmoth *Deilephila elpenor* can learn to discriminate flowers using chromatic information even in starlight, but when given the choice between different intensity versions, it exhibits preferences for darker or brighter shades of the training colour (Kelber et al., 2002). Differences in the achromatic

TABLE 2 | Studies that enable assessing the relative importance of chromatic versus achromatic contrast in the context of flower detection and discrimination.

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
Lepidoptera						
<i>Macroglossum stellatarum</i>	Paper stimuli	D	Naïve animals show a preference for the stimulus with higher intensity	Discrimination task		Present study
<i>Macroglossum stellatarum</i>	Mono-chromatic blue lights	D	Naïve animals show a preference for the stimulus with higher intensity. Achromatic cues are learned, but more slowly than chromatic cues	Discrimination task	In a conflicting situation, the chromatic aspect is given higher weight	Kelber, 2005
<i>Macroglossum stellatarum</i>	Paper stimuli	D	Naïve animals can use both chromatic and achromatic contrast to guide the proboscis to the nectary	Detection task: nectar guide versus surrounding petal colour		Goyret and Kelber, 2012
<i>Deilephila elpenor</i>	Paper stimuli	N	Naïve animals show a preference for the stimulus with higher intensity	Discrimination task		Present study
<i>Deilephila elpenor</i>	Paper stimuli	N	After training to a colour, animals use the chromatic aspect to choose. However, when provided the training colour in several intensities, they prefer brighter shades of yellow and darker shades of blue, suggesting a contribution of achromatic vision	Discrimination task		Kelber et al., 2002
<i>Manduca sexta</i>	Feeder	C	Naïve moths prefer bright white flowers in dim light and with dark background	Detection: Importance of achromatic contrast of the flower to the background increases with decreasing illumination intensity	Both used stimulus colours differed in both achromatic and chromatic aspect of colour; the preference for blue disappears in dim light	Kuenzinger et al., 2019
<i>Manduca sexta</i>	Paper stimuli	C	Naïve animals probed on white lines on a black or dark blue flower background, but avoid black or dark blue lines on a white background, indicating that proboscis control is mediated by achromatic cues independent of the chromatic aspect	Discrimination of nectar guides, as contrast was high enough for detection in all cases		Goyret, 2010
<i>Helicoverpa armigera</i>	Paper	N	Animals appeared to select the most reflective stimuli	Discrimination task	The setup was not ideally designed for testing achromatic vision, as the aim was different	Satoh et al., 2016

(Continued)

TABLE 2 | (Continued)

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
<i>Catopsilia pomona</i>	Paper	D	Naïve butterflies choose equally between three shades of yellow differing in intensity, no effect of achromatic differences. Learning of achromatic cues was not tested	Discrimination task	The butterflies have a preference for blue over all other colours, and blue has low intensity	Balamurali et al., 2020
<i>Papilio xuthus</i>	Paper	D	Achromatic contrast to background is required for the animals to land on a flower	Detection and landing on flower	The contrast seems to be for the broad-band receptor, alternatively for a summed receptor signal of UV, blue, green and red receptors	Koshitaka et al., 2011
<i>Papilio xuthus</i>	Paper	D	After training to a single colour stimulus, animals prefers high reflectance over low reflectance stimuli	Discrimination task		Kinoshita et al., 2011
<i>Papilio xuthus</i>	Paper, neutral density filters	D	Naïve animals prefer the brighter of two stimuli. They can learn to choose the brighter or darker of two stimuli, but learning is slower than for chromatic cues	Discrimination task	Tested with backgrounds of different intensities, the animals can use simultaneous brightness contrast, something rarely tested in insects	Kinoshita et al., 2012
Diptera						
<i>Eristalis tenax</i>	Paper	D	Trained animals preferred stimuli of higher intensity, particularly with yellow hues, independent of the brightness of the learned colour	Discrimination task	The main focus of the study was on chromatic vision	An et al., 2018
Hymenoptera						
<i>Apis mellifera</i>	Red ("bee-black"), blue and green paper	D	Learned behaviour	Discrimination task	Bees discriminated red from other colours (even though it appears as achromatic, black to them) so they must have used achromatic information	Turner, 1910
<i>Apis mellifera</i>	Mono-chromatic and white light	D	Learned behaviour	Discrimination	In dim light, bees choose the stimulus of higher intensity, even after training to the other stimulus	Menzel, 1981
<i>Apis mellifera</i>	Paper stimuli	D	Trained behaviour	Edge detection and landing	Edge detection and landing is colour-blind and requires contrast in the green receptor	Lehrer et al., 1990
<i>Apis mellifera</i>	Paper stimuli	D	Learned behaviour	Detection: For flower cues without chromatic contrast to background, detection threshold (3° angular size) depends on achromatic (green receptor) contrast		Lehrer and Bischof, 1995

(Continued)

TABLE 2 | (Continued)

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
<i>Apis mellifera</i>	Paper stimuli	D	Learned behaviour	Detection	Bees learn achromatic (green receptor) cues at small visual angles (5°), though this is harder for them than learning chromatic cues	Giurfa et al., 1996, 1997; Giurfa and Vorobyev, 1998
<i>Apis mellifera</i>	Paper stimuli	D	Learned behaviour	Both chromatic and achromatic (green receptor) contrast mediate detection of coloured patterns	For large targets ($\geq 15^\circ$), chromatic contrast is most important	Hempel de Ibarra et al., 2000, 2001; Niggebrügge and Hempel de Ibarra, 2003
<i>Apis mellifera</i>	Paper stimuli	D	Learned behaviour	Discrimination	Only achromatic (green receptor) contrast is used for shape discrimination	Hempel de Ibarra and Giurfa, 2003
<i>Apis mellifera</i>	Paper stimuli	D	Learned behaviour	Detection	With achromatic (green receptor) contrast present, grouping of small stimuli improves detection	Wertlen et al., 2008
<i>Apis mellifera</i>	Paper stimuli	D	Learned behaviour	Detection (Experiment 1) and discrimination (Experiment 2) both use achromatic information	Only green receptor contrast explained the detection of orange from dark grey. Both green receptor contrast and chromatic contrast together explained discrimination from other flower colours	Reisenman and Giurfa, 2008
<i>Apis mellifera</i>	Paper stimuli	D	Learned behaviour	Detection: the animals could not detect flowers of 28° extension, if they presented no chromatic contrast to the grey background	Green receptor contrast and summed receptor contrast are not learned in this situation	Ng et al., 2018
<i>Apis cerana</i>	Paper stimuli	D	Learned behaviour	Detection threshold for flowers with achromatic and chromatic contrast is 7.7°, for flowers with only chromatic contrast 13.2°		Meena et al., 2021
<i>Bombus terrestris</i>	Artificial flowers of different sizes	D	Learned behaviour	Detection	When searching for large targets chromatic contrasts are used. For small targets, achromatic (green receptor) contrast is used	Spaethe et al., 2001
<i>Bombus terrestris</i>	Paper stimuli	D	Learned behaviour	Detection	With green receptor contrast present, grouping of small stimuli improves detection	Wertlen et al., 2008
<i>Melipona mondury</i>	Powder mixes	D	Preference in several colour combinations in series, visits to all stimuli were rewarded	Discrimination of two colours, no consistent effect of achromatic cues	Background colour (green or grey) had an effect	Koethe et al., 2016

(Continued)

TABLE 2 | (Continued)

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
<i>Melipona quadrifasciata</i>	Powder mixes	D	Preference in several colour combinations in series, visits to all stimuli were rewarded	Discrimination of two colours, no consistent effect of achromatic cues		Koethe et al., 2016
<i>Tetragonula carbonaria</i>	Paper stimuli	D	Learned behaviour	Detection threshold is similar (visual angle) for stimuli with and without achromatic (green receptor) contrast		Dyer et al., 2016
<i>Tetragonula iridipennis</i>	Paper stimuli	D	Learned behaviour	Detection threshold for target with both chromatic and achromatic contrast is 9°, with only chromatic contrast 11.5°		Jezeera et al., 2021
<i>Tetralonia lerlandi</i>	Paper perianth, real flower		At distances < 30 cm, the male search time for <i>Ophrys</i> dummy flowers with different perianth colours was correlated with achromatic, but not chromatic contrast to the green background	Detection task	At distances > 30 cm, search time was affected by wind speed (olfaction), but not by the visual properties of the perianth	Streinzer et al., 2009

D, diurnal; N, nocturnal; C, crepuscular.

cues of flowers may result in changes in plant reproductive success; for example, nutrient deficiency can lead to lower amounts of floral pigment, which reduces flower chromatic and achromatic contrast (Ausma et al., 2021), and shaded flowers may suffer from reduced visitation by insect pollinators (Ushimaru et al., 2021). Generally, the relative importance of chromatic versus achromatic signals in detection and discrimination of flowers by insect pollinators remains largely unknown, partly because studies on insect vision are greatly biased toward the chromatic aspect (Kelber and Osorio, 2010; Hempel de Ibarra et al., 2014).

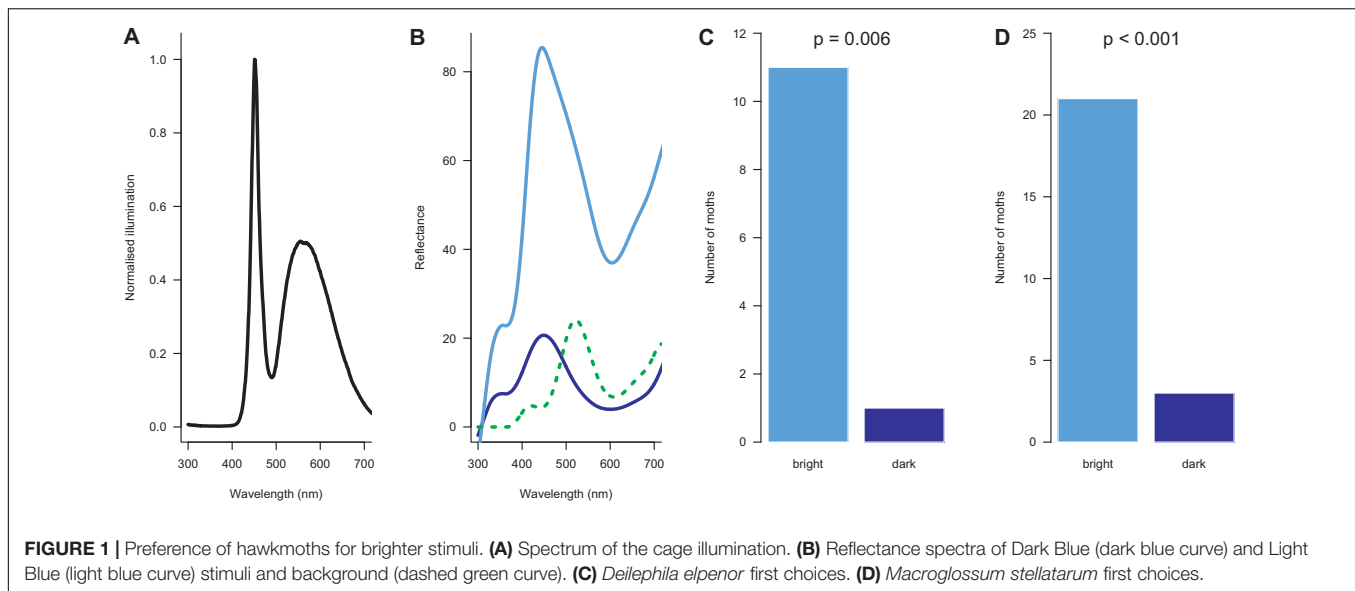
In the following, we take a three-way approach to evaluate the importance of achromatic information for flower detection by flower-visiting insects. We present results from a behavioural experiment on responses of flower-naïve diurnal and nocturnal hawkmoths to stimuli that differ solely in intensity. Thereafter we discuss what is known from literature on the use of chromatic cues by flower-visiting insects. Finally, we use an optical model to better understand the contribution of pigment, scattering structures and type of background for the achromatic contrast between flowers and their background.

BEHAVIOURAL EXPERIMENTS WITH DIURNAL AND NOCTURNAL HAWKMOTHS

Pupae of the nocturnal *D. elpenor* and the diurnal *Macroglossum stellatarum*, bred on the natural larval food plant (*Galium* sp.), were placed in a flight cage at room temperature in a 12:12 h

light:dark cycle for eclosion. Tests were performed in a separate flight cage (65 cm × 65 cm × 80 cm height × depth × width) with three walls from light grey cloth and one long wall from transparent plastic for observation. The cage, placed in an otherwise dark room, was diffusely illuminated from above with an array of LEDs (Goobay LED strip flex 33 SMD white, max 132 lumen) pointing upward toward an aluminium reflector (Figure 1A). The intensity was adjusted to 10 lux for *D. elpenor* (≈nautical twilight) and to 100 lux (≈civil twilight) for *M. stellatarum*, as measured in the centre of the cage (using Hagner Screen Master, Hagner AB Solna Sweden). The two illumination intensities were chosen as similar as possible but also to account for the fact that *D. elpenor* is nocturnal, while *M. stellatarum* is diurnal, but extends its flight into the evening twilight (Herrera, 1992).

The flat visual display, printed on paper, 20 cm high and 28 cm wide, with a green background (spectrum in Figure 2B) and two circular blue flower-like stimuli of 2 cm diameter, in the centre, but 7 cm apart, was presented vertically on a stand about 5 cm in front of the cage wall opposite the wall covered with transparent plastic (for a figure of a similar set-up please see Telles et al., 2014). As the preference of naïve moths was to be tested, we did not provide any reward to the moths. The stimuli, hereafter called Light Blue and Dark Blue, had the same chromatic properties but different intensities (achromatic properties) (Figure 1B). The illumination spectrum (Figure 1A) was measured using a calibrated spectroradiometer (RSP900-R; International Light, Peabody, MA, United States). Reflectance spectra of stimuli and background (Figure 1B) were measured using an integrating sphere and the same setup, following standard routines



(van der Kooi et al., 2016, 2017). The used reference was a diffuse, white tile (Avantes WS-2).

Each newly eclosed, flower-naïve moth was tested once and individually, on the day of eclosion, in their respective activity period (first 2 h of night, for *D. elpenor*, daytime for *M. stellatarum*). A new stimulus array was used for each moth, and the position of Light Blue and Dark Blue (right or left side) was randomised. Hawkmoths do not land on flowers, but approach and probe them with the extended proboscis. Therefore, a single moth was released into the cage and given time to warm up, and the first time it approached and probed one of the stimuli with the extended proboscis was counted as choice. Only the first choice was registered to avoid pseudoreplication. Moths that did not approach and probe the stimulus display within 5 min after taking flight (three *D. elpenor* and four *M. stellatarum*), were considered not motivated to feed and removed. None of the moths probed the green background.

Twelve moths of *D. elpenor* approached the stimulus display with the extended proboscis and probed at least one stimulus. Of these moths, eleven approached Light Blue first, whereas one chose Dark Blue ($p = 0.006$, exact binomial test; 99% confidence interval: 0.52–1.00). Of 24 *M. stellatarum*, 21 probed Light Blue ($p < 0.01$, exact binomial test, confidence interval: 0.61–0.99). In both species, this indicates a significant preference for the stimulus with higher reflectance and intensity (**Figures 1C,D**). A similar preference for the brighter of the two shades of the same colour has earlier been shown for *M. stellatarum* (Kelber, 2005); that study had been performed under higher illumination intensity ($\approx 4,000$ lux) and used (monochromatic) light sources with fairly high intensity differences as stimuli, whereas the stimuli used here are more similar to natural flowers. Although the peak reflectance of the Light Blue stimulus used here is still somewhat higher (80%) than the highest reflectance found in flowers ($\sim 60\%$; van der Kooi et al., 2016, 2017), the strong preference (**Figures 1C,D**) can likely be generalised to natural flowers.

WHAT DOES PUBLISHED LITERATURE SUGGEST ABOUT THE USE OF ACHROMATIC CUES BY INSECTS?

We synthesised the findings of 32 experimental studies that investigated the importance of achromatic versus chromatic aspects of colour in object detection/recognition for in total 15 insect species of bees, butterflies, moths and flies (**Table 2**). We did not list studies that solely investigated chromatic vision, without allowing any insight into the use or lack of use of achromatic cues, and also exclude literature dealing with the use of achromatic or single receptor cues for motion vision or polarisation vision. We also excluded most literature on landing responses, the use of polarised light stimuli and other behaviours that are not directly involved in flower detection or discrimination. We focussed on the use of achromatic cues in the direct context of flower detection and flower discrimination, separating between preferences of flower-naïve (or generally untrained) individuals and individuals that had learned to retrieve a reward from a specific type of flower. As many publications focus on chromatic aspects and mention reactions to achromatic contrast in passing, our list is likely not complete, but it clearly indicates the many ways in which achromatic information is used by different species of pollinators. We posed five questions, and summarise below what we have learned with respect to each of them, also giving some details in **Table 2**.

Do Flower-Visiting Insects Use Chromatic and Achromatic Signals for Separate Purposes as Often Suggested?

It is commonly assumed that insects use chromatic and achromatic cues for different purposes (see “Historical Background”). Specifically for bees, it has been suggested that chromatic cues are important for flower detection and recognition, whereas achromatic channels guide motion vision

and other behaviours. However, this separation may be less complete than previously assumed. Although chromatic cues are learned faster and used more frequently than achromatic cues, achromatic cues can be learned in the context of object – and flower – detection and recognition (Table 2). Several species of moths, flies and bees exhibited an innate preference for high intensity stimuli and/or chose stimuli more (rapidly) in the presence of achromatic cues (Table 2). Only in three species of stingless bees and one tested butterfly species, no effect of achromaticity was found (Table 2).

On the other hand, *P. xuthus* butterflies use both chromatic and achromatic cues for the control of landing (see Table 2), a task which is controlled exclusively by achromatic cues in bees and flies. Whereas landing is an important behaviour for pollination, the literature on landing responses was not included in Table 2, because there is general agreement that insects use achromatic vision for this behaviour (see “Historical Background”). *Macroglossum stellatarum* moths use both achromatic and chromatic cues for finding the entrance to the nectar reservoir (Goyret and Kelber, 2012). Thus, as to be expected from the anatomical and physiological findings that achromatic and chromatic channels are not entirely separated (see “Historical Background”), they both can, at least to some degree, influence the same behaviours to a larger degree than was earlier appreciated.

Is There a Difference Between Diurnal and Nocturnal Insects in the Use of Achromatic Versus Chromatic Cues?

Colour vision and the use of chromatic versus achromatic cues have been studied in fewer nocturnal than diurnal insects. A direct comparison can only be made between the diurnal hawkmoth *M. stellatarum* and the nocturnal species *D. elpenor* (see Figure 1). Although many nocturnal insects, including *D. elpenor* and the nocturnal carpenter bee *Xylocopa tranquebarica* (Somanathan et al., 2008), have (exquisite) colour vision, given the adaptations to increase light capture found in the eyes of nocturnal insects (reviewed by Nilsson, 2021), it is tempting to hypothesize that stimulus intensity plays a role in visibility to many nocturnal insects.

Our behavioural experiments showed that when given the choice between two flowers, naïve individuals express a clear preference for a more reflective, brighter stimulus (Figure 1). The crepuscular hawkmoth *Manduca sexta* was tested with conflicting cues and revealed that under bright light conditions, and with a bright background, they preferred dark blue to white (without UV) flowers, but this changed both with light and background, and in dimmest light levels and with dark backgrounds, the preference clearly shifted to white (Table 2; Kuenzinger et al., 2019).

It has been reported that in honeybees “colour vision disappears” in dim light (Menzel, 1981), but this happens in light intensities when they do not normally fly and forage. Only one nocturnal bee species, the carpenter bee *X. tranquebarica*, has ever been tested for colour vision at night, and this was not done in the context of flower visits, but at the nest

entrance. If we assume that their use of cues is similar in both situations, we could conclude that chromatic information remains more important for that species than achromatic information. Bees trained to associate their nest entrance with a yellow marker, consistently searched at yellow markers, independent of intensity cues, and disregarded markers of other colours even if they matched the intensity of the learned marker (Somanathan et al., 2008).

Based on the limited number of species tested it seems that the use of achromatic information indeed differs between situations and light conditions, but whether there are overall differences between diurnal and nocturnal species remains unclear.

Is There a Difference in How Chromatic and Achromatic Cues Guide the Innate and Learned Preferences of Flower Visitors?

Innate colour preferences are considered to shape foraging behaviour in various insect groups. Both bees and moths have been shown to be able to learn colours other than what they innately prefer (van der Kooi et al., 2019), whereas syrphid flies seem to more strongly adhere to their innate preferences (Lunau and Maier, 1995; An et al., 2018). For the spontaneous preferences of flower-naïve animals, we see clear evidence for the use of achromatic cues in moths (Satoh et al., 2016; Kuenzinger et al., 2019; this study), but not in bees, flies or butterflies (Table 2). Bees, butterflies and moths can learn achromatic cues, but when given a conflicting situation, they will rely more heavily on the chromatic aspect. A tendency to rely on chromatic aspects in conflicting situations is likely directly related to the fact that chromatic information is more stable under changing light conditions. Colour constancy, demonstrated in these groups (Werner et al., 1988; Balkenius and Kelber, 2004) will allow an animal to commonly distinguish a yellow flower from a green background, independent of changes in illumination colour, which can be quite dramatic between shade and sun, and between late twilight and a moonlit night (Johnsen et al., 2006).

Do Chromatic and Achromatic Cues Have Different Roles for the Detection of Flowers Against the Background, and for the Discrimination Between Flowers?

Pollinators have two discrimination tasks to solve: first, they need to discriminate a flower from its background, and also from other flowers, which may not be rewarding. That first task is often referred to as detection. In addition to colour cues, insects can rely on depth cues to discriminate between objects and their background, often using motion parallax, which relies on achromatic contrast between flower and background (see “Historical Background,” e.g., Lehrer et al., 1990). We have not included other references to this in Table 2, as little has been done on flower-visiting insects. However, butterflies require achromatic contrast to land on flowers (Koshitaka et al., 2011), and *M. stellatarum* needs achromatic contours to stabilise

hovering flight in front of a flowers (reviewed by Stöckl and Kelber, 2019).

The detection from the background is a more complex problem, as flowers can be seen in front of many different backgrounds, such as a clear blue featureless sky, a sky with moving clouds, dark green vegetation which can also be moving, and soil or human-made structures of various colours. Most of these backgrounds are different in colour from flowers. Although natural background structures (i.e., leaves, soil, and rocks) are roughly similarly achromatic (Menzel and Shmida, 1993; Ellis et al., 2021), recent studies showed that variation in background colour can determine the salience of flowers to pollinators. Bukovac et al. (2017) modelled flower salience against more than 500 natural backgrounds and found that background colour has the potential to significantly change a flower's colour contrast. A recent study by Finnell and Koski (2021) showed that bee and fly colour preferences for ultraviolet flower markings depends on the type of background, though this preference difference depended on chromatic and not achromatic properties. The findings from Menzel et al. (1997) that desert plants exhibit lower achromatic contrast than plants in the Mediterranean due to differences in background and not flower colour also suggests that achromatic aspects of backgrounds are important. However, the discrimination between neighbouring flowers presented against one background may be more challenging when these flowers have similar colours. The smaller the chromatic or achromatic difference is, the higher sensitivity is needed.

Studies on European honeybees have found that these flower-visitors can both detect (and discriminate) flowers that exhibit achromatic contrast from a three times larger distance (with a subtended visual angle of 5°) than flowers presenting chromatic contrast (15°), and a similar result is found for the Asian honeybee (see **Table 2** for references). A smaller difference has been observed in bumblebees (Dyer et al., 2008; Wertlen et al., 2008) and stingless bees (Dyer et al., 2016; Jezeera et al., 2021). Honeybees use achromatic and chromatic contrast in similar ways for detection of flowers from the background, for discrimination between differently coloured flowers and for discrimination of flower patterns (Hempel de Ibarra et al., 2000, 2001; Niggebrügge and Hempel de Ibarra, 2003). We therefore conclude that achromatic and chromatic information is used for both flower detection and discrimination. Nevertheless, because the achromatic channel often has a higher spatial resolution (see above) it might be more important in the detection task.

How Do Floral Scattering and Pigmentation Properties Determine the Achromatic Contrast?

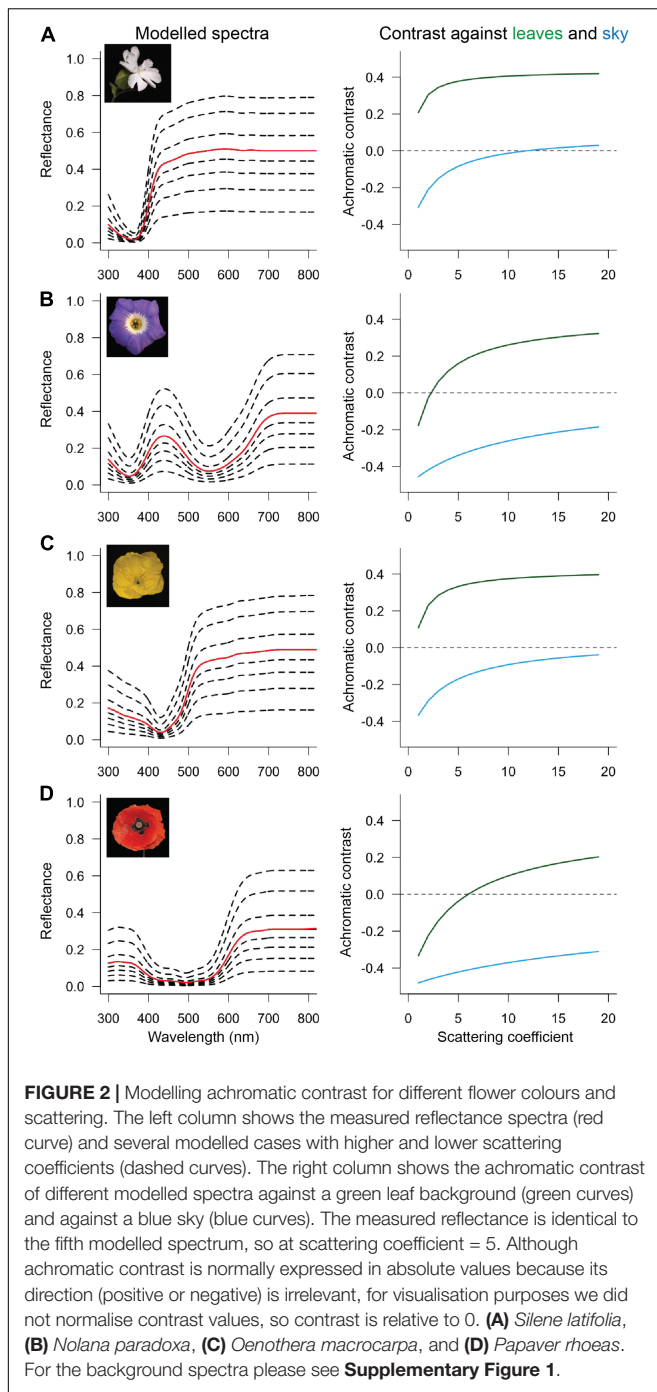
Flowers differ from their background in both the chromatic and achromatic aspect of colour (Chittka et al., 1994; Kevan et al., 1996; Menzel et al., 1997; van der Kooi et al., 2019). It is generally assumed that achromatic contrast is determined by the type of pigment (Kevan et al., 1996; Narbona et al., 2021). For example, white and yellow flowers generally exhibit higher achromatic contrast than blue, purple and red flowers. However, flower colour is not only determined by pigments,

but also by structures that scatter incident light, such as cell walls, starch granules and the flower's surface (van der Kooi et al., 2016; Stavenga et al., 2020). Flowers generally consist of different layers, such as the epidermis, mesophyll and starch layer, which all scatter part of the incident light. The reflectance of a flower increases when individual layers become more inhomogeneous or when the number of layers increases. How the scattering properties of flowers determine the achromatic contrast is largely unstudied.

To gain a more quantitative understanding of how achromatic contrast varies for different types of pigmentation and the amount of scattering, we deployed our previously devised optical model (Stavenga and van der Kooi, 2016). The optical model is based on the Kubelka-Munk theory for scattering and absorbing media and relies on spectral measurements as input. The model enables to systematically investigate the contribution of different optical properties, such as the amount of scattering or pigmentation (van der Kooi et al., 2016, 2017; van der Kooi and Stavenga, 2019). We used a white, blue, yellow and ultraviolet-reflecting red flower (*Silene latifolia-alba*, *Nolana paradoxa*, *Oenothera macrocarpa*, and *Papaver rhoeas*, respectively), systematically varied their scattering coefficient independent from pigmentation properties and calculated the achromatic contrast against a green leaf or blue-sky background (D65, midday). Achromatic contrast was calculated as the von Kries-corrected difference in excitation of the honeybee's long-wavelength (green) photoreceptor between the stimulus and background (**Supplementary Figure 1**), after Spaethe et al. (2001). For details on the modelling and parameter setting, see methods in van der Kooi (2021).

Modelling achromatic contrast as a function of the amount of light scattering revealed clear differences between the type of pigment and backgrounds, though for all modelled cases the response curves are very similar in shape. There is a pattern of diminishing returns for all colours and backgrounds: the strongest changes in achromatic contrast are obtained when the scattering coefficient is low (**Figure 2**). At very high scattering coefficients (> 10), the achromatic contrast curve plateaus for all colours and backgrounds. The scattering coefficients found in flowers in nature varies between ~ 1 and 10 (van der Kooi et al., 2016, 2017), presumably due to mechanical constraints associated with flower thickness and interior inhomogeneity, which are the principal factors that determine backscattering. From the modelling (**Figure 2**) it becomes clear that producing flowers with extremely high backscattering also provides little benefit in terms of achromatic contrast and visibility.

Against a green leaf background, white and yellow flowers exhibit higher achromatic contrast than blue and red flowers (**Figures 2A,C**). The achromatic contrast of yellow and white flowers is also comparatively high regardless of the amount of scattering, because their pigments absorb light over a small wavelength range. Achromatic contrast further increases with scattering but plateaus around 0.4, close to the maximum contrast that is theoretically possible (0.5). The modelled blue and red flowers exhibit low achromatic contrast (**Figures 2B,D**), although this also increases with scattering coefficient. Against a blue sky the opposite occurs:



blue and red flowers exhibit a much higher achromatic contrast than white and yellow flowers, at least with low scattering coefficients, because these colours are comparatively dark against the bright sky. Nevertheless, when flowers are high up a tree or stalk and presented against the sky background, the visual signal for pollinators may also be determined by the transmission instead of the reflection, or a combination of both, which may differ in spectral composition and intensity (van der Kooi et al., 2016).

Our modelling illustrates how flower colour and structure, as well as the type of background determines a flower's achromatic contrast. Overall, the relationship between achromatic contrast and scattering coefficient mimics the response of colour contrast as a function of the amount of pigment (van der Kooi, 2021). For weakly pigmented flowers, an increase in the amount of pigmentation yields a stronger increase in visibility than in flowers that have a moderate amount of pigment (van der Kooi, 2021). Future studies could reveal whether there are trade-offs in visibility via the chromatic versus the achromatic channel, how that translates to different flower pigmentation and anatomy, as well as whether the flower's location on the plant and the type of background are driving factors for the evolution of the optical properties of flowers.

CONCLUDING REMARKS

Research on animal vision traditionally focussed more on the chromatic aspect than the achromatic aspect. Various factors contributed to this bias, e.g., the presumed dichotomy in signal processing between the achromatic and chromatic channel as well as the taxonomic bias toward honeybees as model system. In a changing light environment, chromatic information is more invariant and robust than achromatic information. On the other hand, achromatic cues have a higher signal to noise ratio, particularly in dim light. Presumably therefore, during daytime or in constantly changing illumination conditions, chromatic vision may be more reliable, whereas in dim light, achromatic vision may be more useful than chromatic vision. Our behavioural experiment suggests that naive *M. stellatarum* and *D. elpenor* hawkmoths prefer a higher intensity to a lower intensity stimulus if both stimuli have the same chromatic properties. Various studies tested the detection and discrimination of stimuli with and without achromatic contrast to the background (Table 2). The overall picture is that, overall, chromatic contrast largely determines a stimulus' detectability, particularly at short distances; however, achromatic contrast contributes to visibility both physiologically as well as behaviourally. Achromatic information feeds into the colour vision channel (see "Historical Perspective") and it mediates detection of stimuli by bees, flies, butterflies, and moths (Table 2). For several species-groups, however, it is still unknown which type of photoreceptor is most important for achromatic signal detection (Table 1). Presumably, the use of achromatic cues also plays a role in detecting oviposition sites for herbivorous insects (Prokopy et al., 1975; Blake et al., 2020; Yang et al., 2020).

The finding that achromatic cues are important for flower detection by bees, flies, butterflies and moths has broader relevance for flower colour evolution. Chromatic cues may be most important for diurnal flowers pollinated by bees, butterflies or birds, and achromatic cues may be particularly important for flowers that are pollinated at night. In addition to diurnal and nocturnal hawkmoths that show a preference for stimuli of high intensity (this study), bats use colour-blind rod vision at night (Borges et al., 2016) and thus rely on achromatic visual signals. It has frequently been noted that nocturnal

pollinated flowers are often white or yellow (e.g., Kevan and Baker, 1983), which both appear bright to animals that use the long-wavelength photoreceptor for achromatic vision. Our optical modelling showed that scattering properties crucially determine the achromatic contrast of flowers (section “How Do Floral Scattering and Pigmentation Properties Determine the Achromatic Contrast?”). We therefore propose that not just the pigments, but also the amount of reflected light determines the visibility and brightness of nocturnal pollinated flowers. This means that flowers pollinated at night may evolve structural features that enhance reflectance, e.g., more cell layers or more strongly scattering structures. Further, selective pressures on floral visual signals may be coupled with floral display size. Larger displays will be noticeable from longer distances – and this process is mediated particularly by achromatic vision. Small flowers may thus compensate for their limited visibility via extra reflectance and achromatic contrast. We welcome future experimental studies on pollinator vision and comparative studies on the optical properties of the flowers that they pollinate.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

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AUTHOR CONTRIBUTIONS

Both authors contributed equally to the experiment, wrote this study, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.819436/full#supplementary-material>

Supplementary Figure 1 | The blue and green background spectra used for the model calculations.

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