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RESEARCH ARTICLE

Subtractive colour mixing with bile pigments creates the rich wing palette of *Graphium weiskei* butterflies

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ABSTRACT

The wings of the purple spotted swallowtail *Graphium weiskei* are marked by an unusual bright colour pattern. Spectrophotometry on *G. weiskei* wings demonstrated the presence of a pigment with an absorption spectrum (peak wavelength λ_{max}=676 nm) similar to that of the bile pigment sarpedobilin in the wings of the congeneric *Graphium sarpedon* (λ_{max}=672 nm). Sarpedobilin alone causes cyan–blue wing areas, but the green-coloured areas of *G. sarpedon* wings result from subtractive colour mixing with the carotenoid lutein. Reflectance spectra of the blue-coloured areas of *G. weiskei* wings indicate that sarpedobilin is mixed with the short-wavelength-absorbing papiliochrome II. An enigmatic pigment, tentatively called weiskeipigment (λ_{max}=580 nm), enhances the saturation of the blue colour. Weiskeipigment causes a purple colour in areas where the sarpedobilin concentration is low. The wings of the related papilionid *Papilio phorcas* contain the bile pigment pharcobilin (λ_{max}=604 nm), as well as another sarpedobilin (λ_{max}=663 nm). The cyan to greenish wings of *P. phorcas* are due to phorcabilin and sarpedobilin mixed with papiliochrome II. A survey of known subspecies of *G. weiskei* as well as of congeneric *Graphium* species of the '*weiskei*' group shows various degrees of subtractive colour mixing of bilins and short-wavelength absorbers (carotenoids and/or papiliochromes) in their wings. This study illuminates the underestimated role of bile pigments in butterfly wing colouration.

KEY WORDS: Reflectance spectrum, Pigmentary colouration, Bilins, Sexual dichromatism

INTRODUCTION

The purple spotted swallowtail *Graphium weiskei* is an extraordinarily colourful butterfly species found in mountainous regions of New Guinea, but the optical basis of the colour pattern has not been fully clarified. In an extensive study of the anatomy and colouration of several *Graphium* species, Allyn et al. (1982) reported that at the dorsal (upper) side of the coloured wing areas, sparse piliform scales, setae or bristles have replaced the lamellate scales, common for lepidopterans. They furthermore found that the wings of *G. weiskei*, like those of *G. sarpedon* and *G. agamemnon*, are coloured by pigments concentrated in granules in the wing membrane (Allyn et al., 1982).

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The pigmentation of the blue and green midband of *G. sarpedon* wings is well known. The main pigment is the bile pigment sarpedobilin (Choussy and Barbier, 1973; Barbier, 1981). Sarpedobilin has an absorption spectrum peaking at ∼670 nm with a substantial sideband only in the ultraviolet and thus will create a cyan colour when present in an inhomogeneous, scattering medium. Yet, whereas a restricted wing area of *G. sarpedon* indeed has a cyan colour, the main part of the wing midband has a distinctly green colour. There, sarpedobilin is mixed with the carotenoid lutein, which acts as a blue-absorbing colour filter (Allyn et al., 1982; Rothschild and Mummery, 1985; Stavenga et al., 2010).

Previously, the bile pigment pterobilin was first isolated from cabbage butterfly wings (of no less than 10^6 specimens provided by Austrian schools; Wieland and Tartter, 1940). It was found to be identical to the pigment of blue–green caterpillars and pupae, which was identified as the tetrapyrrole biliverdin-IXγ (Rüdiger et al., 1968). Subsequently, two related bilins were identified by Choussy, Barbier and colleagues, who performed a series of extensive studies on the complex chemistry of the bile pigments in several butterfly species (Choussy et al., 1973; Choussy and Barbier, 1973; Bois-Choussy, 1977; Barbier, 1981). Focusing on the papilionids *Papilio phorcas* and *Graphium sarpedon*, their specific bile pigments, called eponymously phorcabilin and sarpedobilin, were demonstrated to be formed by cyclisation of the basic pterobilin (Bois-Choussy and Barbier, 1983). Whereas pterobilin was demonstrated to exist in the wings of numerous investigated papilionids, nymphalids and pierids, it was found only in some saturniid moths and not in sphingids (Bois-Choussy, 1977). Furthermore, whereas phorcabilin appeared to be the principal blue pigment in caterpillars and/or wings of several saturniids, sarpedobilin was only found to be the main pigment in the wings of *G. sarpedon* and *G. weiskei* (Bois-Choussy, 1977). From the wings of male *P. phorcas*, mainly phorcabilin, and some sarpedobilin as well as pterobilin, was extracted (Bois-Choussy, 1977).

Allyn et al. (1982) stated that all three bile pigments were present in the wings of *G. weiskei*, an 'unusually beautiful species', which displays bright pink–purple, green and blue patches (Fig. 1A). On the basis of reflection spectrophotometry, while applying the standard extraction procedure for bile pigments on *G. weiskei* wings, Barbier (1983) concluded that only sarpedobilin was present. Barbier (1983) had difficulty extracting the red pigment that Allyn et al. (1982) reported to exist in the intermembranal matrix of the purple wing patches, but after extensive chemical approaches, he concluded that the remaining pink colour was due to ommin, bound to high molecular compounds in the wing membrane (Barbier, 1983). Barbier (1990) also investigated *G. stresemanni*, which has predominantly blue-coloured dorsal wings (Barbier, 1990). The main pigment extracted from *G. stresemanni* wings was identified as the dimethyl ester of sarpedobilin (Barbier, 1990).

Here, I revisit the pigmentary colouration of the *Graphium* species as well as *P. phorcas*, applying spectrophotometric

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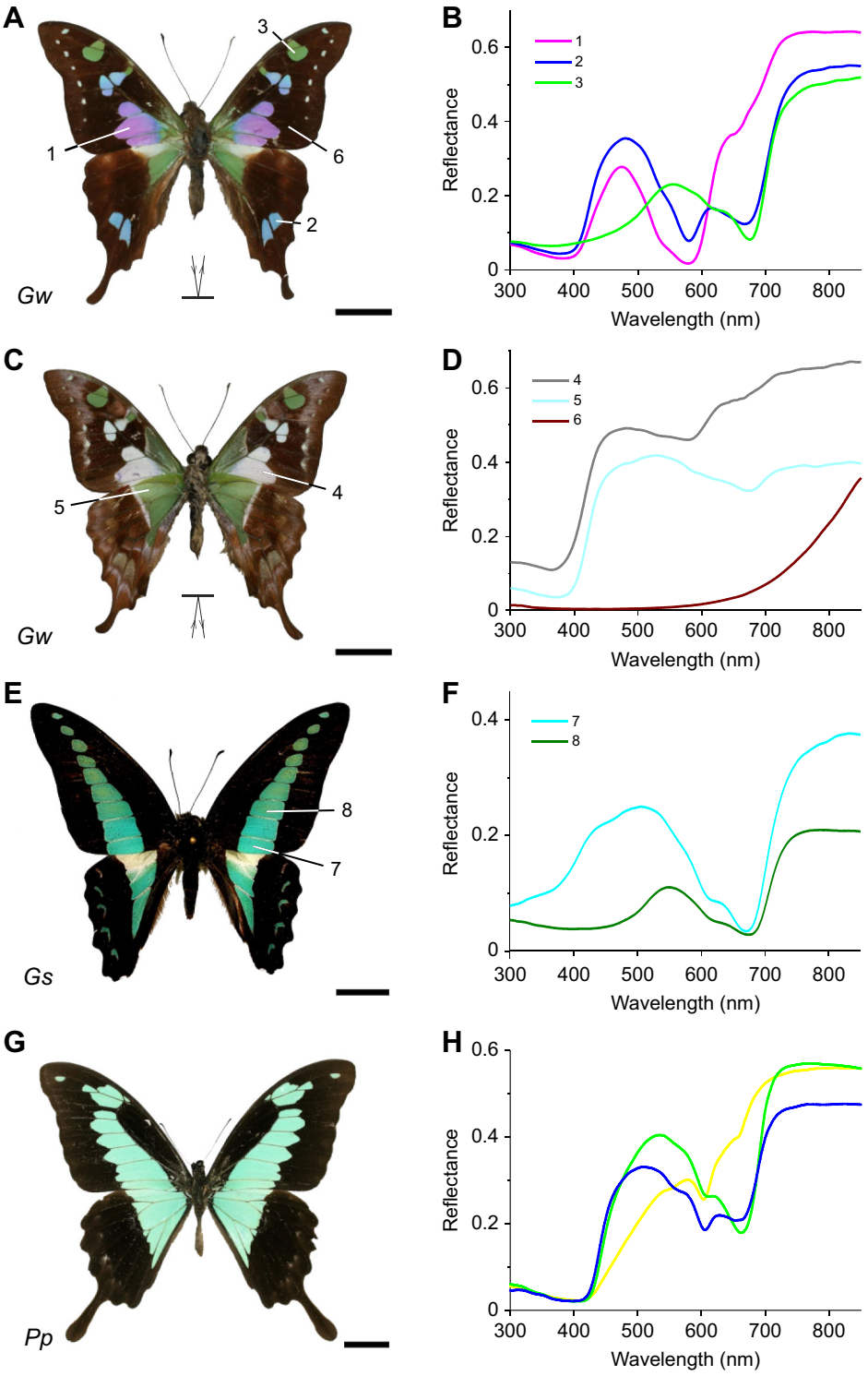

Fig. 1. Colouration and reflectance spectra of *Graphium weiskei*, *Graphium sarpedon* and *Papilio phorcas* wings. (A,B) Dorsal wing side of *G. weiskei* (*Gw*; A) and reflectance spectra of purple, blue and green dorsal wing areas 1, 2 and 3 (B). (C,D) Ventral wing side of *G. weiskei* (C) and reflectance spectra of whitish and pale-green ventral wing areas 4 and 5, and of the brown–black dorsal wing area 6 (D). (E,F) Dorsal wing side of *G. sarpedon* (*Gs*; E) and reflectance spectra of dorsal forewing areas 7 and 8 (F). (G,H) Dorsal wing side of *P. phorcas* (*Pp*; G) and reflectance spectra of different *P. phorcas* specimens (H). Scale bars: 1 cm.

methods. I conclude that the different colours are due to subtractive colour mixing of a number of bile pigments, carotenoids and papiliochromes. The chemical nature of the various pigments needs further study.

MATERIALS AND METHODS

Specimens and photography

Specimens of *Graphium weiskei* Ribbe 1900, *Graphium stresemanni* Rothschild 1916 and *Papilio phorcas* Cramer [1775] were purchased from de museumwinkel.com (https://www.demuseumwinkel.com/). *Graphium sarpedon* (Linnaeus 1758) was captured in Hayama, Japan. The butterflies were photographed with a Canon EOS 70D digital camera.

Spectrophotometry

Wing reflectance spectra were measured with a bifurcated probe connected to a halogen/deuterium light source and an Avantes AvaSpec-2048-2 CCD detector array spectrometer (Avantes,

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Apeldoorn, The Netherlands). The reference was a white diffuse standard (Avantes WS-2). Transmittance spectra of small areas ($10\times10\ \mu m^2$) of wing pieces were measured with a microspectrophotometer (MSP), consisting of a Leitz Ortholux microscope with a LUCPlanFL N 20×/0.45 objective (Olympus, Tokyo, Japan) and the Avantes spectrometer. The wing pieces were embedded in immersion oil to eliminate most of the reflection and scattering on the inhomogeneities of the wing scales and membrane.

RESULTS

Colouration of *Graphium weiskei*

The dorsal wings of *G. weiskei* are marked by bright pink–purple, blue and green patches within a main brown–black background (Fig. 1A). Close-up examination shows that the coloured patches of the dorsal wings are devoid of scales. The colouration is therefore not due to scales, which is the usual colour mechanism of butterflies, but to a pigmented wing membrane. Reflectance spectra of the coloured dorsal-wing patches (Fig. 1A, areas 1–3) measured with a bifurcated reflection probe show a low reflectance in the ultraviolet wavelength range (300–400 nm; Fig. 1B), demonstrating that the scales contain an ultraviolet-absorbing pigment, most likely papiliochrome II (Wilts et al., 2012; Stavenga et al., 2015). The valleys in the spectra suggest the presence of additional pigments with absorption maxima around 580 and 670 nm (Fig. 1B).

On the ventral wing side, the green areas only have a sparse set of thin, hairlike scales, while the white patches are covered by white, unpigmented scales (Fig. 1C). The latter also holds for the row of subterminal forewing spots that are blue when observed at the dorsal side. The hindwing patches that are dorsally blue are ventrally covered by brown, melanised scales. Consequently, of the ventral wings, only the green areas still stand out, because of their sparse cover of slender scales. Reflectance spectra of the whitish-coloured, ventral-wing patches (Fig. 1C, areas 4 and 5) show the same valleys around 580 and 670 nm (although much less distinct) as are seen in the reflectance spectra of the dorsal wing areas (Fig. 1B,D). This indicates a major contribution to the reflectance by the scattering ventral-wing scales. The reflectance spectrum of the brown–black wing areas (Fig. 1A, area 6) has the classical spectral shape of melanised scales (Fig. 1D; Stavenga et al., 2014). As the valleys at ∼670 nm in the spectra of Fig. 1B,D resemble those in the reflectance spectra previously obtained from the wings of *G. sarpedon* (Stavenga et al., 2010), this triggered a revisit to that case.

Colouration of *Graphium sarpedon*

The reflectance spectra of dorsal forewing areas 7 and 8 of *G. sarpedon* (Fig. 1E; called Df1 and Df3 in Stavenga et al., 2010) both have a valley at ∼670 nm, due to the absorption by sarpedobilin, but the spectra differ distinctly in the short-wavelength range (Fig. 1F). The wing reflectance spectra (7, 8) of *G. sarpedon* do not show a distinct reflectance valley near 400 nm as in Fig. 1B (1, 2). The latter was indicative of the UV-absorbing papiliochrome II, but the lower reflectance of the green area with respect to that of the cyan patch of *G. sarpedon* (Fig. 1F) is due to the carotenoid lutein that is contained in area 8 but not in area 7 (Stavenga et al., 2010).

Comparing the spectra of *G. sarpedon* with those of *G. weiskei* shows a clear correspondence in reflectance minima near 670 nm (Fig. 1B, 2, 3; Fig. 1F, 7, 8). At this wavelength there is a minor kink in the spectrum of the purple wing area of *G. weiskei* (Fig. 1B, 1). Evidently, the purple area has only a minor sarpedobilin concentration. Remarkably, the reflectance spectrum of the blue wing area of *G. weiskei* has a sharp, additional dip near 580 nm (Fig. 1B, 2), which is even much more prominently visible in the spectrum of the purple area (Fig. 1B, 1). According to Barbier (1983), the responsible pigment is an ommin. However, the only ommin for which a (limited) spectrum has been published is ommin A, isolated from shrimp eyes, which has an absorbance peak wavelength at 520 nm (Butenandt et al., 1958a). These puzzling spectral characteristics suggested further information must be obtained, by first studying the properties of pharcobilin.

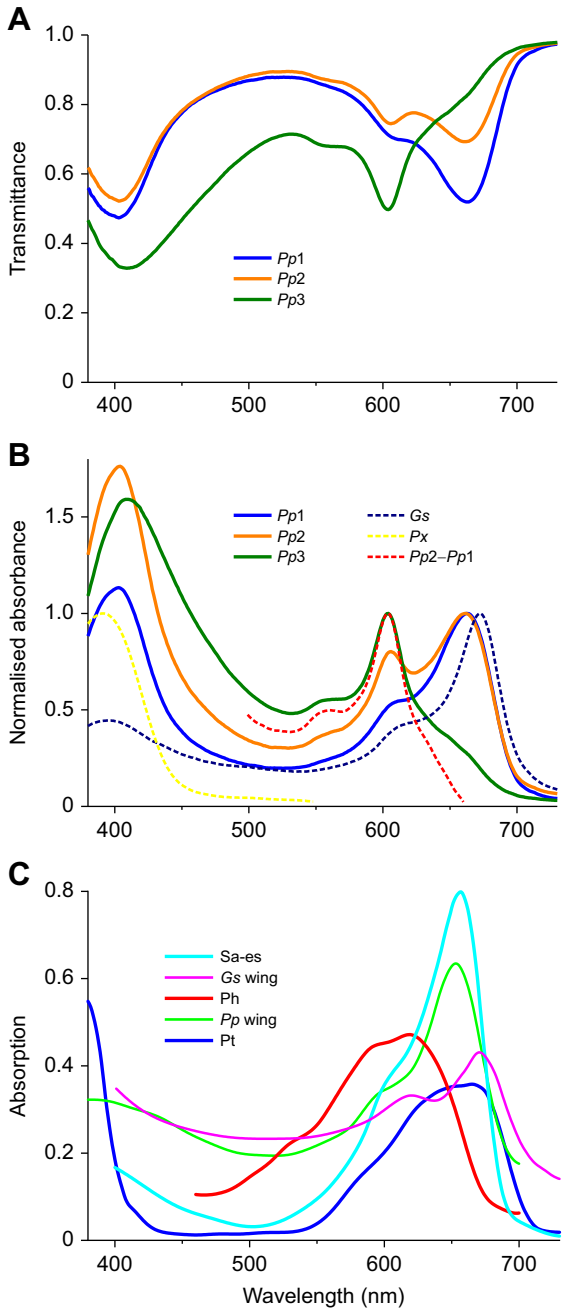

Fig. 2. *Papilio phorcas* wing spectra compared with literature data. (A) Transmittance spectra measured with a microspectrophotometer on three wing parts of *P. phorcas* (*Pp*1–3) immersed in immersion oil. (B) Absorbance spectra calculated from the transmittance spectra normalised at the long wavelength peak. *Gs* is the normalised absorbance spectrum of sarpedobilin of *Graphium sarpedon* (from fig. 7B in Stavenga et al., 2010). *Px* is the absorption spectrum of the yellow pigment of *Papilio xuthus* (from fig. 1D in Stavenga et al., 2015). (C) Absorption spectra reported by Bois-Choussy (1977); see Results. Sa-es, sarpedobilin ester; Ph, phorcabilin; Pt, pterobilin.

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Colouration of *Papilio phorcas*

The wing colouration of *P. phorcas* butterflies can vary, from cyan–green (as in the specimen shown in Fig. 1G) to distinctly green and even yellow, the latter notably in some females (Rothschild and Mummery, 1985; see also https://collector-secret.proboards.com/thread/160/papilio-dardanus-subspecies-forms). The coloured wing areas are studded with well-developed scales, quite different from the *Graphium* species, where the scales in the coloured areas have a reduced, piliform shape. The pigments underlying the colouration of *P. phorcas* exist in both scales and wing membrane. Fig. 1H shows a few reflectance spectra measured from different specimens. The low reflectance in the ultraviolet wavelength range (Fig. 1H) is virtually identical to that of areas 1 and 2 of *G. weiskei* (Fig. 1B), indicating the action here again of papiliochrome II. Well recognisable is also a valley near 670 nm, and the spectra show an additional dip, near 600 nm, distinctly different from that near 580 nm of *G. weiskei*.

To better characterise the pigments, I embedded wing pieces in immersion oil and measured the transmittance with a microspectrophotometer (Fig. 2A, *Pp*1–3). Fig. 2B shows the three transmittance spectra converted into absorbance spectra, normalised at the long-wavelength peaks. The shape of the long-wavelength parts of the spectra suggests that they are due to a mixture of two components. *Pp*1 presumably represents the first component, because its spectral shape is very similar to the previously determined absorbance spectrum of sarpedobilin of *G. sarpedon* (Stavenga et al., 2010). However, whereas the absorbance peak wavelength of sarpedobilin of *G. sarpedon* is λ_{max}=672 nm, for *P. phorcas* sarpedobilin, λ_{max}=663 nm (Fig. 2B). Concerning the second component in the *P. phorcas* spectra, subtracting spectrum *Pp*1 from *Pp*2 with subsequent normalisation (Fig. 2B, *Pp*2–*Pp*1) yields a spectral band similar to spectrum *Pp*3, except for the latter's larger tail. For the absorbance peak wavelength of the second component of *P. phorcas* (*Pp*2–*Pp*1), apparently pharcobilin, it thus follows that λ_{max}=604 nm (Fig. 2B). In the short-wavelength range, the absorbance spectra of *Pp*1–3 show a smooth band, without the characteristic peaks of carotenoids, but very similar to that of papiliochrome II, measured in *Papilio xuthus* (Fig. 2B, *Px*; Stavenga et al., 2015).

Before returning to the wing colouration of *G. weiskei*, it may be useful to consider the data reported by Bois-Choussy (1977), because she investigated the chemistry of the butterfly wing pigments in great detail. Unfortunately, the reported spectral data are confusing. Fig. 2C recollects a few relevant absorption spectra measured in extracts (*in vitro*) and wings (*in situ*) (taken from figs 27–29 of Bois-Choussy, 1977). The spectrum for sarpedobilin ester in MeOH/H^+ extracted from *G. sarpedon* wings shows a distinct absorption peak in the far-red, λ_{max}=655 nm, with low absorption in the visible wavelength range (Fig. 2C, Sa-es). The spectrum measured on *G. sarpedon* wing fragments (Fig. 2C, *Gs* wing) was stated to peak at 670 nm, which is in good agreement with the λ_{max}=672 nm derived for sarpedobilin in our subsequent work (Stavenga et al., 2010), but the spectrum shows a considerable absorption throughout the visible wavelength region (Fig. 2C, *Gs* wing). This was possibly caused by the ample presence of melanin in *G. sarpedon* wings. The spectrum reported for phorcabilin in pH 7.2 buffer (Fig. 2C, Ph), extracted from *P. phorcas* wings, is very broad and is stated to have the same peak wavelength, λ_{max}=655 nm, as the sarpedobilin ester. However, the spectrum of the extract strongly deviates from the *in situ* measured wing spectrum, which is very sarpedobilin-like (Fig. 2C, *Pp* wing). Furthermore, the spectrum for pterobilin in pH 7.2 buffer (Fig. 2C, Pt), which was isolated from *Pieris brassicae* wings (Bois-Choussy, 1977), is included here. It differs from the spectrum in the original pterobilin study (Wieland and Tartter, 1940). The conclusion of the widely deviating spectra must be that for adequate understanding of the local wing colours, more reliable spectral data are necessary.

Pigmentation of *G. weiskei*

The green colouration of *G. sarpedon* that is caused by a mixture of lutein and sarpedobilin is an example of subtractive colour mixing. This insight was used to further analyse the pigmentation of *G. weiskei*. Microspectrophotometry of small wing parts, embedded in immersion oil, revealed the differences in local pigmentation. Fig. 3A,B shows transmittance spectra of wing pieces of *G. sarpedon* and *G. weiskei* converted into absorbance spectra; the numbers of the spectra correspond to the wing locations of Fig. 1A,E. Normalisation of spectra 3, 7, 8 of Fig. 3A,B at their absorbance maximum (~670 nm) yields the spectra of Fig. 3C. Spectrum 7 of Fig. 3C represents the absorption spectrum of the sarpedobilin in *G. sarpedon* wings. The difference spectrum of spectra 8 and 7 of Fig. 3C, shown normalised in Fig. 3E (*Gs*), represents the absorption spectrum of lutein (Stavenga et al., 2010). The normalised difference spectrum of spectra 3 and 7 of Fig. 3C (Fig. 3E, *Gw*) represents a blue-absorbing pigment that together with sarpedobilin causes the green-coloured patches in *G. weiskei* wings. The pigment clearly differs from lutein, but the minor oscillations in the spectrum indicate that it is a mixture of lutein (and possibly other carotenoids) and papiliochrome.

The absorbance spectrum of *G. weiskei*'s green wing area has a long-wavelength band with peak wavelength λ_{max}=676 nm (Fig. 3F, *Gw* Sa), showing that this band is due to a sarpedobilin, related to that of *G. sarpedon*, for which λ_{max}=672 nm (Fig. 3F, *Gs* Sa) and *P. phorcas* sarpedobilin, which was found above to have λ_{max}=663 nm (Fig. 3F, *Pp* Sa). *Graphium weiskei*'s sarpedobilin obviously causes the long-wavelength tail of the absorbance spectra of the purple (Fig. 3B, 1) and blue (Fig. 3B, 2) wing patches, but both spectra have a distinct peak at 580 nm. To isolate the pigment responsible for the latter peak, Fig. 3D shows spectrum 1 together with a reduced spectrum 2, so that the spectra are equal at 676 nm. The difference spectrum of spectra 1 and 2 of Fig. 3D is shown normalised in Fig. 3F (*Gw* We). Its absorbance peak wavelength, λ_{max}=580 nm, clearly differs from that of phorcabilin, derived from *P. phorcas*, which has λ_{max}=604 nm, but the spectral peak shapes are very similar (Fig. 3F, *Pp* Ph). The pigment of *G. weiskei* with λ_{max}=580 nm is tentatively called here weiskeipigment.

Alike the absorbance spectra of the immersed wing pieces (Fig. 3B, 1 and 2), the reflectance spectra of the purple and blue patches of *G. weiskei*'s dorsal wings (Figs 1B and 3B, 1 and 2) show the presence of both sarpedobilin and weiskeipigment. The kink at ~670 nm in reflectance spectrum 1 of Fig. 1B clearly indicates that the amount of sarpedobilin in the purple wing patches is small. The concentration of sarpedobilin is evidently much larger in the blue and green patches (Figs 1B and 3B, 2 and 3). As with the green patches, the distinctly blue coloured areas result from the subtractive colour mixing of different pigments. Maybe not surprisingly, whereas the sarpedobilin and weiskeipigment alone create a cyan and purple colour, respectively, a mixture of more or less equal amounts of the pigments causes a deep-blue colour (Fig. 1A, area 2).

Colouration of *G. stresemanni*

This finding triggered a closer examination of *G. stresemanni*, which has wings that display prominent blue-coloured wing areas (Fig. 4). Reflectance spectra measured with the bifurcated probe of

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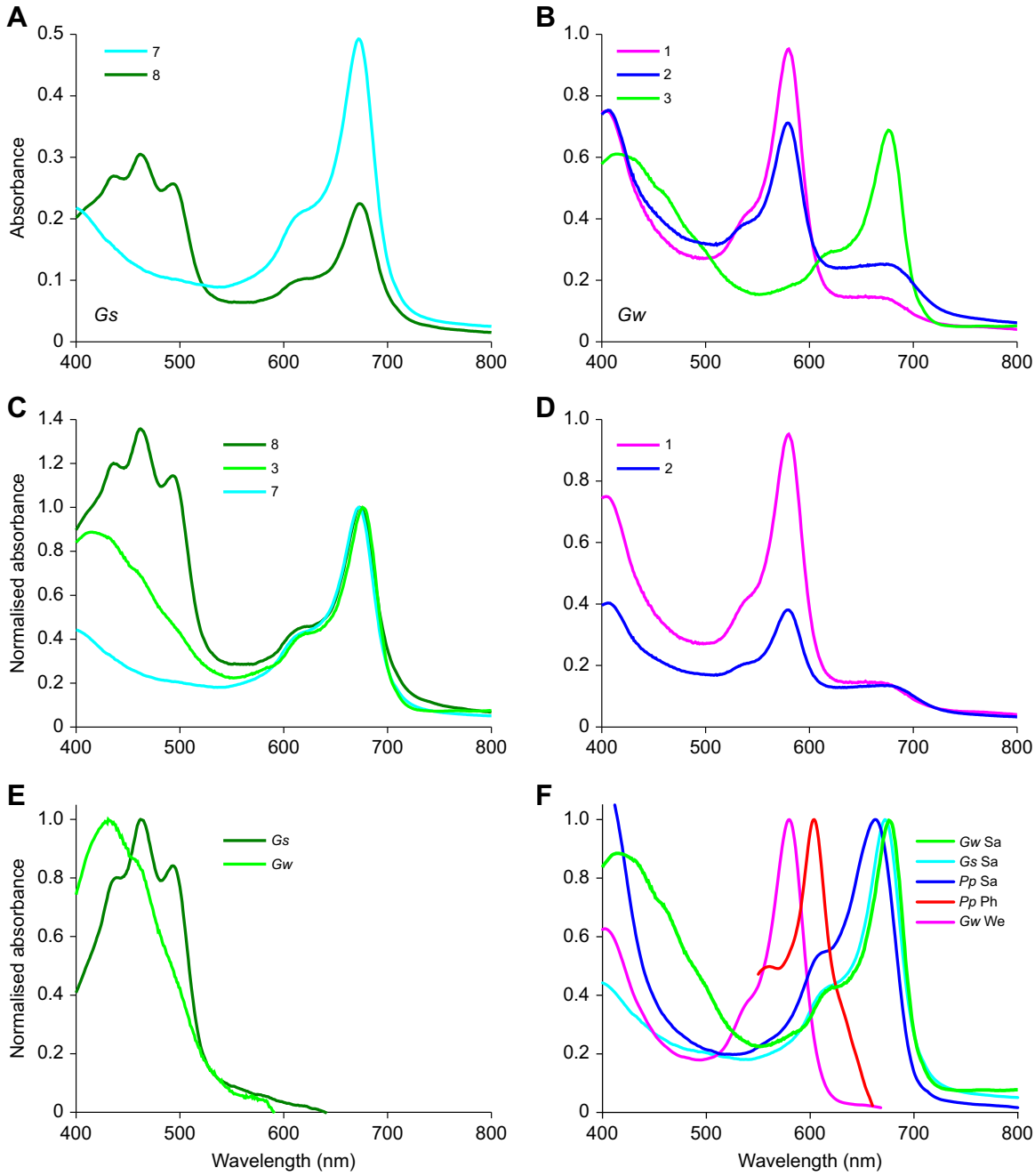

Fig. 3. Carotenoid and bile pigment spectra derived from transmittance measurements of oil-immersed wing pieces of *G. sarpedon* and *G. weiskei*. (A) Absorbance spectra of *G. sarpedon* (*Gs*) dorsal forewing areas 7 and 8 (see Fig. 1E). (B) Absorbance spectra of *G. weiskei* (*Gw*) dorsal wing areas 1, 2 and 3 (see Fig. 1A). (C) Absorbance spectra 3, 7 and 8 of A and B normalised at the long wavelength peak. (D) Absorbance spectrum 1 of B, normalised at peak wavelength 580 nm, and absorbance spectrum 2 of B, adjusted to have the same value as spectrum 1 at 676 nm. (E) Normalised difference spectrum of spectra 8 and 7 of C (*Gs*), and that of spectra 3 and 7 of C (*Gw*). (F) Absorbance spectra derived for *G. weiskei* (*Gw*), *G. sarpedon* (*Gs*) and *P. phorcas* (*Pp*) of the pigments sarpedobilin (Sa), phorcabilin (Ph) and weiskeipigment (We) normalised at their long-wavelength peak. The short-wavelength range contains contributions of carotenoid and papiliochrome pigment.

the blue patches of *G. weiskei* (Fig. 1B, 2) and *G. stresemanni* (Fig. 4B, 1 and 2) are strikingly similar. Clear valleys at 670 and 580 nm thus convincingly demonstrate that the blue areas of *G. stresemanni* wings also contain both sarpedobilin and weiskeipigment, together with UV-absorbing papiliochrome. The green wing patches of *G. stresemanni* (Fig. 4B, 3; Fig. 4D, 6) have clearly the same pigmentation as those of *G. weiskei* (Fig. 1B, 3). Furthermore, the ventral wings of *G. stresemanni* and *G. weiskei* are very similarly patterned and pigmented (Figs 1C,D and 4C,D).

Subtractive colour mixing by bile pigments in *Graphium* species

The insight gained into the pigmentation and colouration of *G. sarpedon*, *G. weiskei* and *G. stresemanni* is helpful to understand the variation in the colour patterns displayed by the two sexes of *G. weiskei* as well as by known subspecies and other, congeneric butterfly species. For instance, Fig. 5A–D shows the males of four subspecies of *G. weiskei*, Fig. 5G,H shows two female *G. weiskei*, and Fig. 5E shows *G. batjanensis*. Fig. 5F,I shows male

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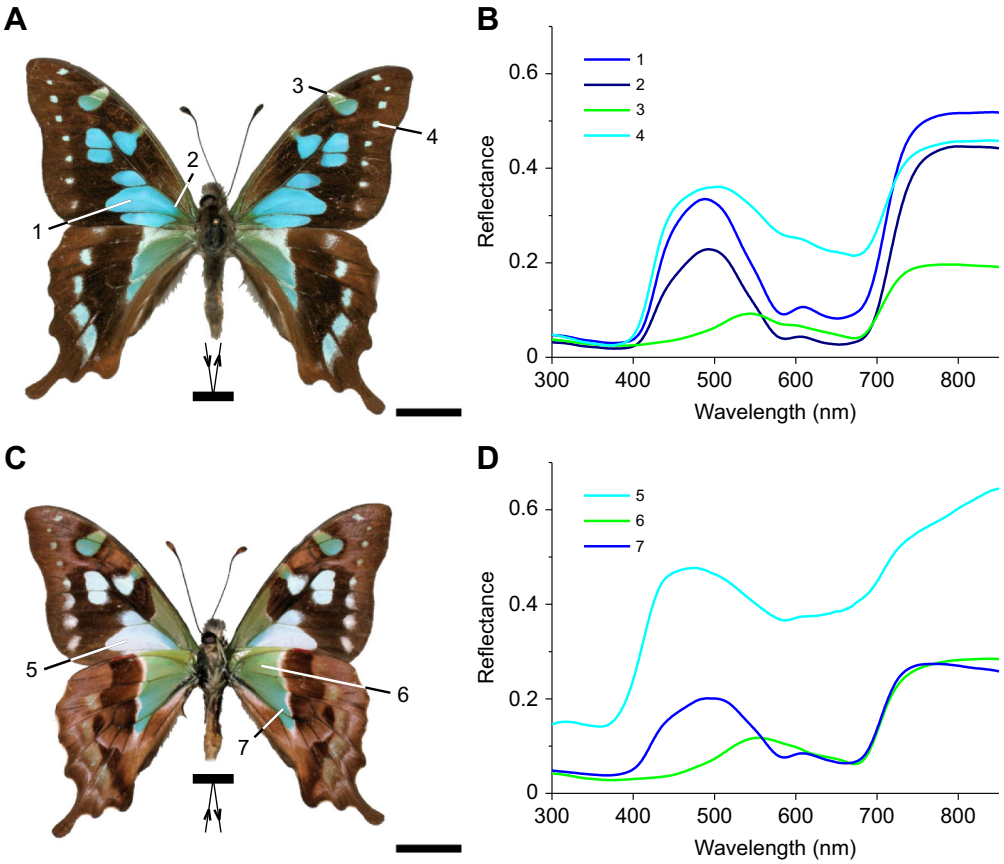

Fig. 4. Colouration and reflectance spectra of *Graphium stresemanni* (Seram Island). (A) Dorsal wing side. (B) Reflectance spectra of blue and green dorsal wing areas 1–4. (C) Ventral wing side. (D) Reflectance spectra of whitish, pale-green and blue ventral wing areas 5–7.

and female *G. kosii*, another *Graphium* member of the '*weiskei*' group. All species of this group, which also comprises two closely related species, *G. macleayanus* and *G. gelon* (not shown), are restricted to the Australasian region, living generally in and adjacent to mountainous areas or islands (Müller and Tennent, 1999). *Graphium weiskei* thus may form an intriguing biogeography case study.

The wing patterning is very similar in all cases, but the colouration of the dorsal wings can differ in considerable detail, especially in male versus female *G. weiskei*. The subapical patches of the forewing and the subbasal areas of the hindwing display about the same green colour. The females *G. weiskei* excel in showing bright pink–purple wing patches. This demonstrates the substantial expression of weiskeipigment, together with a very low concentration of sarpedobilin compared with the males (Fig. 5G,H). The prominently blue *G. batjanensis* (Fig. 5E) resembles *G. stresemanni* (Fig. 4A), which may be related to their habitats being at two not too distant islands of the Moluccan archipelago. The case of *G. kosii* is quite deviant, as the forewing submedian patches are green, obviously due to the combined action of sarpedobilin and carotenoids and/or papiliochrome, and the yellow hindwing submedian patches indicate that the short-wavelength-absorbing pigments are dominant there. The striking sexual dichromatism of *G. weiskei* (Fig. 5A,G) is not apparent in *G. kosii,* however (Fig. 5F,I).

DISCUSSION

Spectrophotometry on the butterflies *G. weiskei*, *G. sarpedon* and *P. phorcas* demonstrated that various bile pigments together with carotenoid and papiliochrome pigments make their wings quite colourful. The wing membrane segments of *G. sarpedon* with a cyan colour contain only sarpedobilin, but the colour of the segments that also contain lutein is bathochromic shifted towards the green wavelength range (Stavenga et al., 2010), as a result of subtractive colour mixing. The green colour of the subapical patches of the forewing of *G. weiskei* (Fig. 1A, area 3) as well as that of the submedian area of the hindwing may be due to the presence of a mixture of various carotenoids (Fig. 3E), in line with the reported occurrence of several carotenoids in the same butterfly species (Rothschild and Mummery, 1985). The low wing reflectance in the UV is most probably due to strongly UV-absorbing papiliochrome II.

Previously, the chemical characteristics of the wing pigments of *G. sarpedon*, *G. weiskei*, *G. stresemanni* and *P. phorcas* were analysed in extracts (Bois-Choussy, 1977; Barbier, 1983). In these species, the degree of expression of sarpedobilin was found to vary, but a possible spectral species dependence was not reported. The sarpedobilin, identified as sarpedobilin ester in both *G. sarpedon* and *G. weiskei*, is bound to chromoproteins within the wing membrane (Barbier, 1990). Applying spectrophotometry of the pigments *in situ*, this study showed that the absorbance spectra of the pigments in the investigated species slightly differ. The species-dependent spectral characteristics suggest binding to different proteins. This may also explain the substantial differences noted between the species in the ease of extracting the sarpedobilin from the wings of *G. sarpedon* versus *G. weiskei* and *G. stresemanni* (Barbier, 1990).

Graphium stresemanni, which has been debated to be a subspecies of *G. weiskei*, but on the basis of a full row of terminal spots on the hindwing is treated as a distinct species, has a series of green and deep-blue spots on the dorsal wings (Barbier, 1990). In line with the abundant deep-blue colouration, the extracts of *G. stresemanni* wings yielded double the amount of sarpedobilin

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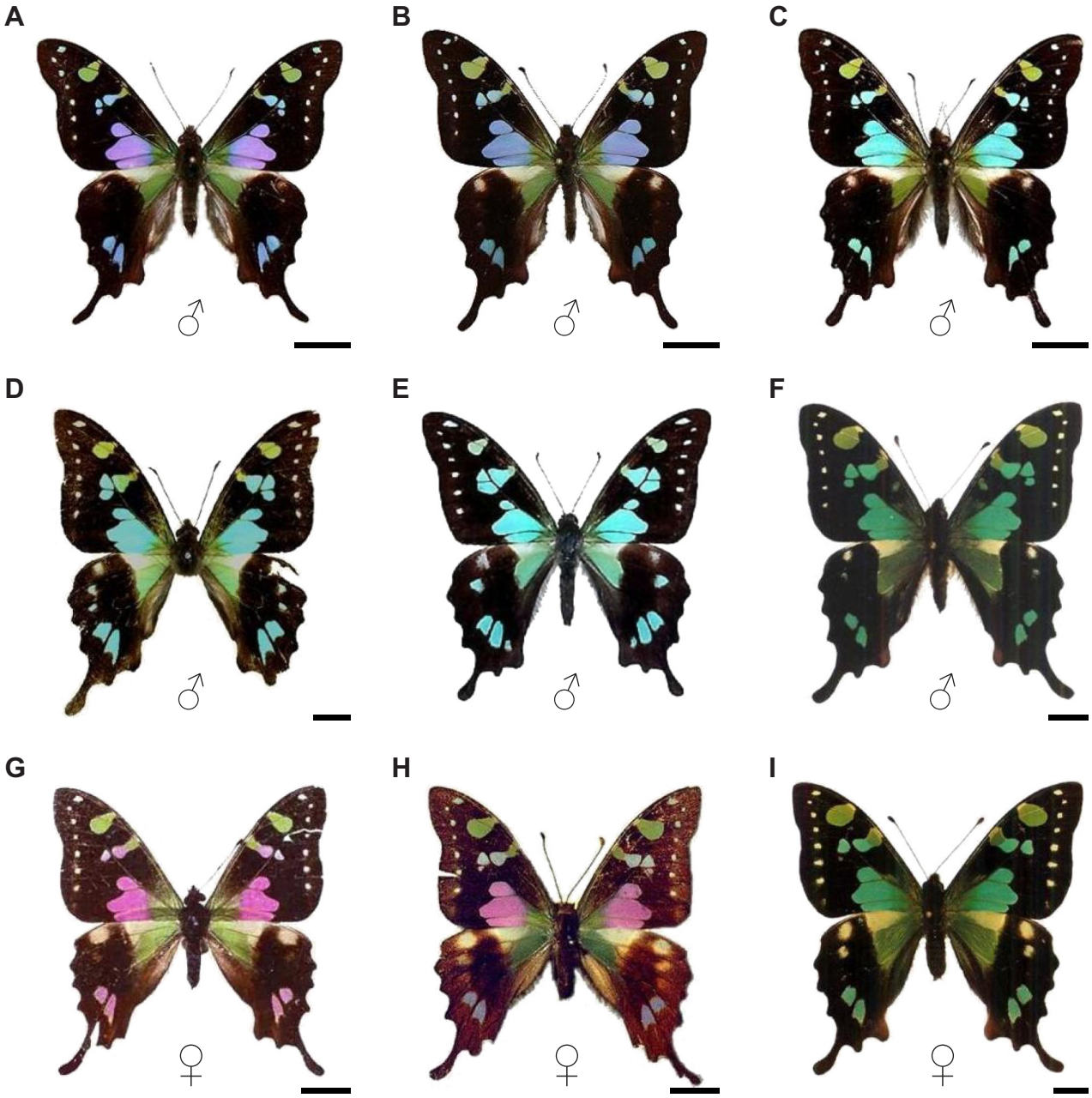

Fig. 5. Butterflies of the *G. weiskei* group living in New Guinea and adjacent islands. (A) *Graphium weiskei weiskei* (Pass Valley, Suwagi). (B) *Graphium weiskei weiskei* (Star Mountains, Batimban). (C) *Graphium weiskei weiskei* (Star Mountains, Arfak). (D) *Graphium weiskei goodenovii* (Goodenough Island). (E) *Graphium batjanensis* (Bacan Island). (F) *Graphium kosii* (New Ireland). (G) *Graphium weiskei weiskei* (Simbu Province). (H) *Graphium weiskei weiskei* (*Weyland* Mountains). (I) *Graphium kosii* (New Ireland). A–F, males; G–I, females. Scale bars: 1 cm. A–C,H were derived from https://www.papua-insects.nl/insect%20orders/Lepidoptera/Papilionidae/Graphium/weiskei/Graphium%20weiskei.htm; E was from https://www.worthpoint.com/worthopedia/graphium-batjanensis-unmounted-butterfly; and D,F,I,G were from published papers (Müller and Tennent, 1999; Braby and Armstrong, 2001; Tennent and Mitchell, 2017).

obtained from *G. sarpedon* and *G. weiskei* wings, but curiously only the extraction of sarpedobilin was reported for *G. stresemanni* (Barbier, 1990). This is strange, because spectrophotometry shows that the deep-blue wing patches of *G. stresemanni*, as well as those of *G. weiskei*, contain abundant weiskeipigment together with sarpedobilin (Fig. 4).

The weiskeipigment when expressed in the wing without being combined with sarpedobilin creates the purple wing patches of *G. weiskei*. The purple colour was attributed to a red pigment (Allyn et al., 1982), which Barbier (1983) after extensive chemical treatments concluded to be an ommin. The present spectral data seem to conflict with this conclusion, however, because, as noted above, the absorbance peak wavelength of ommin A (520 nm), isolated from shrimp eyes (Butenandt et al., 1958a; Linzen, 1974), widely differs from that of weiskeipigment (580 nm). Furthermore, ommins have been encountered predominantly in arthropod eyes, including the papilionid *Papilio machaon*, and the skin of cephalopods, but they could not be demonstrated in insect wings (Butenandt et al., 1958b); the uninvestigated *G. weiskei* could be an exception, of course. Ommochromes generally have quite broad-

band absorbance spectra, very different from the spectrum of the weiskeipigment deduced here (Figs 2B and 3F). The long-wavelength absorption band of weiskeipigment closely resembles that of the sarpedobilins (see Fig. S1). An alternative explanation could therefore be that the weiskeipigment is a modified state of sarpedobilin, which upon extraction coalesces with sarpedobilin. If the weiskeipigment is indeed another bilin, it then could well be called weiskeibilin. Evidently, the chemical nature of the weikeipigment needs further study (see also Butenandt et al., 1967).

In wing areas with only weiskeipigment, the resulting colour is purple/pink. With only sarpedobilin, a cyan colour results, but adding an increasing amount of weiskeipigment will progressively suppress the red part of the reflectance spectrum, causing a deep blue colour. The weiskeipigment thus performs an alternative method of subtractive colour mixing, opposite to that of the carotenoid lutein.

The purple wing areas are backed ventrally by a cover of colourless scales. They have a dual role, first to support the camouflage function of the ventral wing side, and second to enhance the dorsal colour saturation by backscattering light incident from the dorsal side after it has passed the wing membrane.

The wing colouration of *Graphium* butterflies depends not only on the pigmentation but also on the fine-structure of the wing membrane. In *G. sarpedon*, the dorsal surface is covered with minute lens-like papillae, which in *G. weiskei* are sharply tapered (Allyn et al., 1982). The dimensions of the papillae are in the sub-micrometre range, which was exploited in a surface-enhanced Raman scattering study on protein binding (Garrett et al., 2009). The papillae resemble the nanoprotuberances of the nipple array of the transparent wings of the hawkmoth *Cephonodes hylas*. This array acts as a very effective antireflection device (Yoshida et al., 1997), but the interdistance of the papillae of *G. weiskei* wings is substantially larger than that of the moth wing nipples. The pointed papillae will nevertheless create a roughening of the surface that will reduce the wing surface reflectance and thus enhance the saturation of the wing colour (Kilchoer et al., 2019). Note furthermore that the bile pigments have been demonstrated to be concentrated in pigment granules in the intermembrane matrix, which in dead (and thus dried) butterflies are solid. In living animals, the pigments are in a fluid phase in the haemolymph of the wing veins (Allyn et al., 1982), and thus the colouration may somewhat differ between the dead and alive state. Yet, the pigments also occur in the piliform scales of *G. sarpedon* (Stavenga et al., 2010) and the normally shaped scales of *P. phorcas*.

The general view on the colouration and patterning of the dorsal wings of butterflies is that they crucially function for display while the ventral wing pattern serves for camouflage. The dorsal wing pattern of *G. weiskei* is indeed strikingly colourful, and the ventral chequered pattern (Fig. 1C), which consists of prominent green and brown areas, will easily melt the butterfly into the natural background. Indeed, the ventral colour pattern, which is very similar for all species of the '*weiskei*' group, often closely matches the environment (see, for example, the habitat photograph of *G. weiskei* on www.papua-insects.nl/insect%20orders/Lepidoptera/Papilionidae/Graphium/weiskei/Graphium%20weiskei.htm). Yet, the pigmentation is moderate, so that the wings of the species studied here are rather translucent, which may enhance the butterflies' display during flight (Stavenga et al., 2023).

Incidentally, whereas the green colour of *G. sarpedon*'s wings is due to carotenoid mixed with sarpedobilin, very similarly, the green colour of the background leaves is due to carotenoid mixed with chlorophyll. The latter pigment is also a tetrapyrrole and has an absorption spectrum resembling that of sarpedobilin. Finally, *G. weiskei* presumably also possesses the rich spectral set of visual photoreceptors that was discovered in *G. sarpedon* (Chen et al., 2016), which will be well suited to discriminate the colour patterns of the conspecifics and habitat. The wing pattern of *G. weiskei* thus is well designed for intraspecific display and survival, and is as well a delight for the human observer.

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Competing interests
The author declares no competing or financial interests.

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Data availability
All relevant data can be found within the article and its supplementary information.

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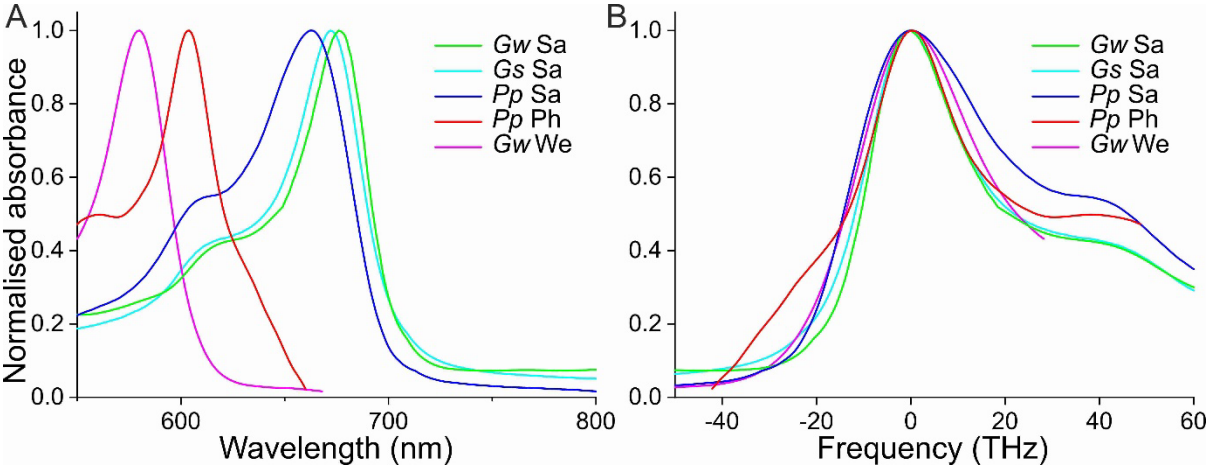

Fig. S1. Absorbance spectra of sarpedobilin (Sa), phorcabilin (Ph) and weiskeipigment (We) obtained for *G. weiskei* (*Gw*), *G. sarpedon* (*Gs*) and *P. phorcas* (*Pp*). **A** The spectra as a function of wavelength normalised at their long-wavelength peak. **B** The spectra as a function of the difference of frequency from the peak frequency, showing a similar spectral band shape. The deviations of the two *P. phorcas* pigments, of phorcabilin (*Pp* Pp) at the long-wavelength side and of sarpedobilin (*Pp* Sa) at the short-wavelength side, are presumably due to mutual contaminations.