

## ON THE USE OF ULTRAVIOLET PHOTOGRAPHY AND ULTRAVIOLET WING PATTERNS IN BUTTERFLY MORPHOLOGY AND TAXONOMY

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**ABSTRACT.** In a series of feeding experiments we found that, depending on the larval food plant species or part of food plant ingested, individuals of the blue butterfly *Polyommatus icarus* (Lycaenidae) exhibit broad variation of wing patterns in the ultraviolet (UV) range of wavelengths which is invisible to humans. Such intraspecific variability in UV wing patterns has been underestimated thus far due to the rather demanding approach needed to study these patterns. We discuss methodological problems with the assessment of butterfly UV wing patterns by UV photography. Given proper standardization, UV photography is a suitable method to qualitatively assess UV wing patterns for possible use in morphology or systematics. Spectrophotometry should preferably be used as quantitative method when considering UV wing patterns in a communication context. No higher value should be attached to UV wing patterns as compared to human visible wing patterns.

**Additional key words:** *Polyommatus*, ultraviolet light, visual communication, color, phenotypic plasticity.

Ultraviolet (UV) wing patterns have been widely used in butterfly systematics. Differences in UV reflectance patterns of butterfly wings proved helpful in the revision of otherwise morphologically very similar taxa, such as the genera *Colias* (Ferris 1973, Silberglied & Taylor 1973, 1978, Kudrna 1992) and *Gonepteryx* (Nekrutenko 1964, Kudrna 1975, Brunton et al. 1996). Differing UV wing patterns were suspected to act as “isolating mechanisms” between closely related species, e.g., by Meyer-Rochow (1991) in *Lycaena*, or shown to be involved in mate choice of several species (e.g., Silberglied & Taylor 1973, 1978, Rutowski 1977, 1981).

Butterflies, in general, perceive UV light and UV vision is an integral part of their visual capabilities (Eguchi et al. 1982, Silberglied 1984, Lunau & Maier 1995, Tovée 1995, Kelber & Pfaff 1999). The same is true for many visually guided butterfly predators such as birds, lizards, and robberflies (Menzel & Backhaus 1991, Jacobs 1992, Fleishman et al. 1993, Tovée 1995). Therefore, UV coloration of butterfly wings has to be considered as an essential part of overall wing patterns in the spectral range of 300 nm to 700 nm, i.e., the entire range of visual communication (Endler 1990, Cuthill & Bennett 1993, Bennett et al. 1994b). Human observers cannot perceive UV light directly. This may be the reason why many researchers implicitly or explicitly attached an extraordinary meaning to the coloration of butterfly wings in the UV range of wavelengths as compared to the human visible range. In the New Zealand *Lycaena salustius* (Lycaenidae) species complex, for example, distinction of species based on

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human-visible wing patterns and morphology of genitalia is possible (Gibbs 1980), yet the discovery of marked differences in UV reflectance patterns was taken even as evidence to suggest the existence of UV wing pattern-based isolating mechanisms (Meyer-Rochow 1991; for another case study in the genus *Lycaena* see Schaidler (1988) versus van Oorschot & de Prins (1989)). Human lack of UV perception, and the processing of visual stimuli in other animals by nervous systems which are completely different from our own, make it very hard for the researcher to imagine what the world may look like for other animals. But it seems as if the lack of UV vision in humans and many other mammals is the exception rather than the rule in the animal kingdom (cf. Tovée 1995).

In this paper we compare UV photography and spectrophotometry as methods for assessing butterfly UV wing patterns. In particular, we discuss some methodological problems with the assessment of butterfly UV wing patterns by UV photography. Finally, we point out an underestimate of individual variability in UV wing patterns which may result from such methodological problems as well as from the neglect of environmentally driven phenotypic plasticity.

### MATERIALS AND METHODS

**Feeding experiments.** Mated females of *Polyommatus icarus* (Rottemburg, 1775) (Lycaenidae) were caught in summer 1997 at two locations in Northern Bavaria, Germany and allowed to lay eggs. Caterpillars used in this study were from the F1 or F2 generations of these females. All larvae were reared in the same climate chamber (25°C, 18 h light, 6 h dark). We kept the larvae in transparent plastic boxes (125 ml) lined with moist pa-

per tissue. Fresh food plant material was available *ad libitum* and was supplied at least every two days. Food plants were either grown outside in a garden (*Medicago sativa* L. leaves) or collected locally from natural populations (flowers of *Trifolium repens* L., flowers and leaves of *Lotus corniculatus* L.) (all Fabaceae). We killed the emerging butterflies with either HCN or by deep freezing. We measured spectral reflectance of the wings of these specimens and took UV photographs.

**Ultraviolet photography.** UV photographs were taken with a Pentax Asahi Ultra-Achromatic-Takumar 85 mm/f 4.5 UV transmitting lens and Novoflex Auto-Bellows on Agfapan APX 100 film. To exclude all but ultraviolet light a combination of 3 mm Schott UG1 and 2 mm Schott BG38 filters was placed in front of the lens for UV photographs. Illumination was provided by two Metz Mecablitz 45 CT1 flash lights in manual mode so that the same amount of light was available for every exposure. Emission spectra of these flash lights reach far enough into the UV range for the purpose of this study (data not shown). Photographic processing and development of film material was standardized as much as possible. Films were developed for 5 min in 10% Agfa Neutol and fixed for 4 min in Tetenal fixing agent at 21°C.

To examine the effect of using different grades of paper on the appearance of prints to be used for comparing UV patterns, we produced prints on different grades of Agfa Broviro Speed glossy paper. These prints were then developed for 3 min with 10% Agfa Neutol and fixed for 15 min with Tetenal fixing agent at 21°C.

For purposes of comparison we took color photographs of all objects on Kodak Elite II 100 slide film. We used the same equipment as above but without the filters and with one flash light only.

**Spectrophotometry.** We measured wing reflectance with a L.O.T. Oriol spectrometer system (InstaSpec II diode array detector, MS 125 spectrograph with 400 l/mm grating, sighting optic) equipped with a Zeiss Ultrafluor 10/0.20 UV transmitting objective at a right angle to the wing surface. Measuring spot diameter was 0.2 mm, numerical aperture of the measuring beam was 0.14. The measuring spot was illuminated at an angle of 45° to the wing surface via liquid light guide by an Osram XBO 75 W/2 OFR lamp powered by a L.O.T. Oriol 68806 power supply. Numerical aperture of the illumination was 0.08. Wings were oriented so that they were all illuminated from the same apical direction. With this setup we were able to record spectral reflectance of individual wing spots in

the range of 300 nm to 700 nm with a resolution of approx. 0.5 nm. A Spectralon™ 99 reflectance standard was assumed as having 100% reflectance. For further details of methods see Knüttel and Fiedler (2001).

## RESULTS

UV patterns strongly varied in both sexes of the European common blue butterfly, *Polyommatus icarus* (Figs. 1, 2). We found consistent differences of UV reflectance among individuals that were fed different plant species or plant parts during their larval stages. Reflectance in the UV was much lower for animals reared on flowers of *Trifolium repens* and flowers or leaves of *Lotus corniculatus*, as compared to animals reared on leaves of *Medicago sativa* (Figs. 1, 2). These differences were most pronounced in the white spots (as seen with human eyes) but were apparent in the underside ground coloration, too (Fig. 1). Overall, judging from the UV photographs (Fig. 1), one might be tempted to assign highly UV-reflecting specimens reared on *M. sativa* foliage to a different 'species'. No differences in UV reflectance were found for the uppersides, the orange spots, and the black spots (Knüttel & Fiedler 1999).

Altering photographic processing had a strong effect on the appearance of the resulting prints, a phenomenon well known to any photographer. The influence of using photographic paper of differing grades is illustrated in Fig. 3. Even if processed from the identical negative using the same chemicals, the resulting prints of UV photographs may be quite different. We therefore included a calibrated gray scale, made from thick chromatography paper and dyed with various dilutions of black India ink, in every photograph. Spectral reflectance of the steps of the gray scale is illustrated in Fig. 4. Parts of a given photographic print that are of similar brightness, compared to the gray scale, will have a similar reflectance value (Figs. 1 and 3).

The differences in wing patterns in the UV range, among individuals reared on different plant species or plant parts, emerged from UV photographs and spectrophotometric measurements alike. However, more subtle or gradual host plant-dependent color differences could better be visualized in the reflectance spectra (Fig. 2). For example, only by studying the spectra is one able to identify the wavelength ranges where individuals reared on *M. sativa* foliage converge into the variation seen in individuals fed other food plants. Moreover, the small but consistent differences between food treatments in the human-visible range were also only noticeable using spectrophotometric measurement data.

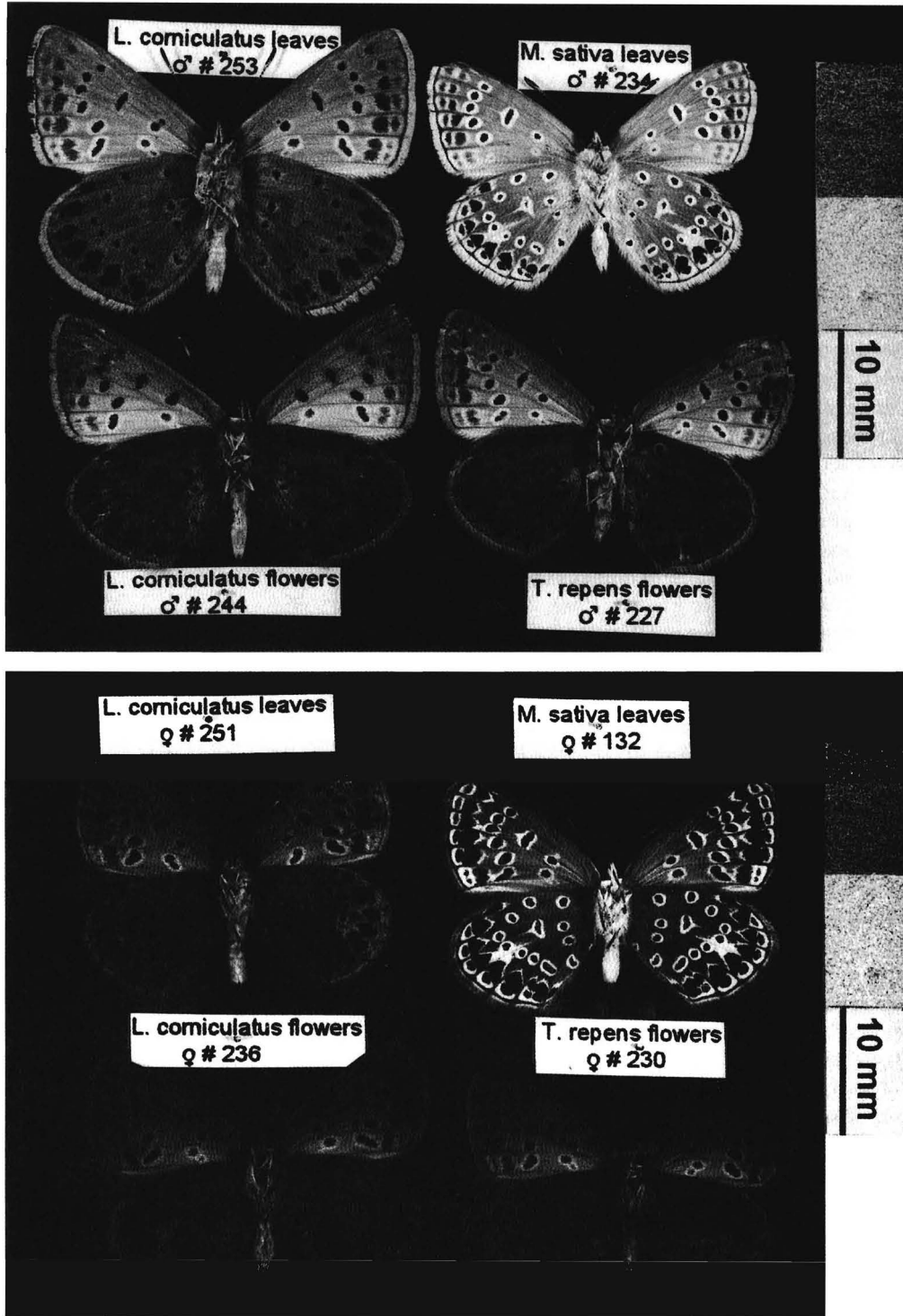


FIG. 1. UV photographs of the undersides of *Polyommatus icarus* reared on different food plants. Differences in UV patterns seen in these individuals are representative for larger series. Individuals were reared on leaves of *Lotus corniculatus* (upper left), leaves of *Medicago sativa* (upper right), flowers of *Lotus corniculatus* (lower left), and flowers of *Trifolium repens* (lower right). *Upper photograph*: males, *lower photograph*: females.

## DISCUSSION

## 1. Underestimate of individual variability in UV coloration

Butterfly systematists are well aware of the large extent of intraspecific variation in wing patterns and coloration within the human visible spectral range. In contrast, intraspecific variability in UV reflection patterns has been underestimated so far. We feel that this mainly arises from the difficulty in studying UV wing patterns, making the comparison of large series of specimens more laborious and demanding compared to patterns seen in the human visible range. Since in most studies UV photography applied to small samples was used to assess UV wing reflectance, subtle differences in UV reflectance of individuals may have frequently been missed.

Though only studied for a small range of butterfly species thus far, intraspecific variation of UV patterns may be as pronounced as, or even larger than that in other ranges of wavelengths visible to humans (Brunton & Majerus 1995, Knüttel & Fiedler 1999, 2001, this work). Yet, minor differences in UV patterns have been sometimes taken as evidence for erecting new species or subspecies (e.g., Nekrutenko 1968, Schaidler 1988, but see van Oorschot & de Prins 1989), or as a later confirmation of taxonomic hypotheses originally proposed on the grounds of other data (e.g., Meyer-Rochow 1991, Coutsis & Ghavalas 1996).

Differences in UV wing patterns may be due to genetic or environmental reasons, but only genetically determined UV wing patterns are of systematic importance. We demonstrated that high intraspecific variation in UV wing patterns in *Polyommatus icarus* can be caused by different larval food plants under otherwise identical rearing conditions among individuals from the same parents.

Flavonoids are a class of secondary plant compounds that highly absorb UV light (Harborne 1991, 1999). Some *Polyommatus* species sequester flavonoids from their larval food plants, and these pigments are deposited in the wings during metamorphosis (Wilson 1987, Wiesen et al. 1994, Geuder et al. 1997, Kornmaier 1999). Using artificial diets which only differed in their flavonoid content but were otherwise identical in their chemical composition, Knüttel & Fiedler (1999, 2001) showed that flavonoids sequestered by the larvae alter wing reflectance mainly in the UV range. In the polyphagous *P. icarus* the types and amounts of flavonoids that are taken up and stored by the larvae vary strongly depending on the larval food plants (Wiesen et al. 1994, Burghardt et al. 1997, 2001, Schittko et al. 1999). Therefore, it seems very likely

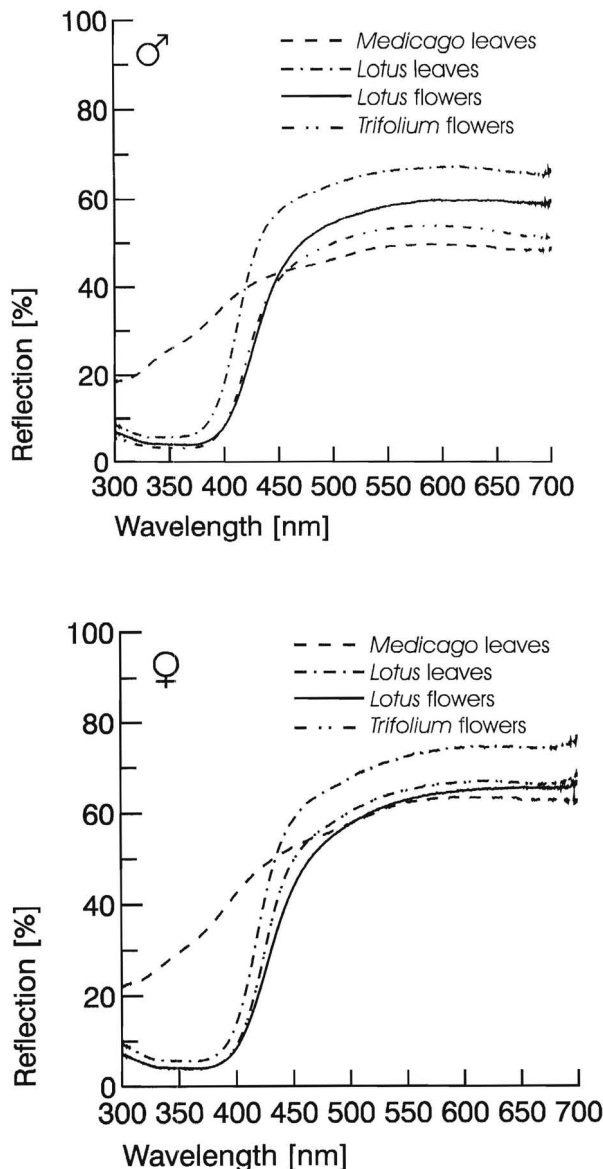


FIG. 2. Spectral reflection of the white spots of the undersides of the hindwings of *Polyommatus icarus* reared on different food plants. Each curve is the mean of the measurements of 5 to 10 spots of a hind wing of one of the individuals illustrated in Fig. 1. Individuals were reared on leaves of *Lotus corniculatus* ( \_ . ), leaves of *Medicago sativa* ( \_ \_ ), flowers of *Lotus corniculatus* ( \_ ), and flowers of *Trifolium repens* ( \_ \_ . ). Upper part: males, Lower part: females. Conventions as in Fig. 1.

that flavonoids are involved in mediating variation in UV wing patterns of *P. icarus* feeding on different food plants in nature.

It is important to emphasize that intraspecific variability in UV reflectance in *P. icarus* appears to be caused by chemical variation in the host plants, while

variation in UV reflectance may be caused by structural colors in other species (e.g., *Colias eurytheme* (Brunton & Majerus 1995)). UV pattern variation in *P. icarus* therefore must be regarded as a host-plant derived, environmentally shaped form of phenotypic plasticity, although heritable components cannot be fully excluded. As different food plant species or plant parts are of varying value as a food source to *P. icarus*, it seems likely that UV wing patterns may be used in intraspecific visual communication, indicating other food plant-derived properties of individuals, such as nitrogen content (Burghardt et al. 2001). Males of *P. icarus* discriminate between flavonoid-rich and flavonoid-free female dummies, preferring UV-absorbing, flavonoid-rich dummies (Burghardt et al. 2000, Knüttel & Fiedler 2001).

The differences shown here in the UV photographs (Fig. 1) and quantitatively demonstrated in the accompanying reflectance spectra (Fig. 2) very much resemble the differences in UV reflectance claimed by Coutsis & Ghavalas (1996) as characters separating *Polyommatus icarus* and the recently described *P. andronicus* Coutsis & Ghavalas, 1995. As differences of such magnitude can be found within one species and even among offspring of the same parents, they are unlikely to be sufficient to differentiate between species. No quantitative data on spectral wing reflectance are available for *P. andronicus*, and the range of individual variation has not been documented statistically. Therefore we cannot presently assess whether significant differences in UV patterns may exist between *P. andronicus* and *P. icarus*. However, based on the UV photographs in Coutsis & Ghavalas (1996) it seems unlikely that UV reflectance in *P. andronicus* falls outside the range observed in the highly variable species *P. icarus*.

## 2. Problems related to the technical visualization of UV patterns

As humans cannot see ultraviolet light, UV wing patterns must be translated into a form of information that is accessible to us. This must be accomplished by appropriate technical means. UV photography or UV videoviewing was chosen in most studies of UV wing patterns known to us (e.g., Ferris 1973, Rutowski 1977, 1981, Bowden & Kay 1979, Meyer-Rochow 1991, Kudrna 1992, Coutsis & Ghavalas 1996). Both methods yield comparable spatial pattern information but almost no spectral information. Ultraviolet light from a broad range of wavelengths is reduced to a single brightness value for every point or pixel in the picture. Usually the spectral response of the picture-generating system is unknown. Alternatively, wing re-

fectance can be measured by spectrophotometry (Ghiradella et al. 1972, Endler 1990, Brunton & Majerus 1995).

Both UV photography and spectrophotometry have advantages and disadvantages in their practical use. When selecting a method to study UV wing patterns the first step must be to answer the questions "What is the purpose of the study? What is it that UV patterns should actually tell me?" Not all studies have adequately addressed these questions. However, different conclusions may have to be drawn from the use of different methods. Therefore it is important to be clear about the purpose of the study before choosing the method.

UV patterns may be considered as a morphological feature like any other character. UV patterns result from wing areas that differ from each other in UV reflectance due to their physical and chemical constitution. There is no conceptual difference to the reflections or colors in the human-visible range. Therefore, UV wing patterns may be used as regular morphological characters in systematics, if they are assessed appropriately. For example, if individuals within a species exhibit substantial variation in UV wing patterns, such as we found in *P. icarus*, then UV wing patterns may not be appropriate systematic characters. UV photography done in the right way (see below) seems a perfectly acceptable means for the description of UV wing patterns in this context.

On the other hand, UV wing patterns may serve as signals in a behavioral context. They may be important in mimetic or aposematic coloration (e.g., Beccaloni 1997) or in sexual selection (e.g., Brunton & Majerus 1995), to give examples. But it is not sufficient to simply assume that UV patterns do have a function, for example in mate recognition. This has to be proven in separate studies reaching farther than assessing differences in UV reflectance only. When considering the visual physiology of butterflies (e.g., Eguchi et al. 1982) or other visually guided species interacting with butterflies (e.g., Bennett et al. 1994a) it seems likely that UV light is important in the species' interactions. But this is so only because UV sensitivity is an integral part of these species' visual systems and is not a consequence of some putative special quality of UV light or vision in the UV range. The mere possibility that UV patterns serve a function in communication gives them no special or "higher" value in systematic reasoning (see e.g., Meyer-Rochow 1991, Brunton et al. 1996). The same is true when comparing UV patterns to human-visible color patterns.

To emphasize this point: There is no reason at all to assign a higher value to UV patterns than to human-

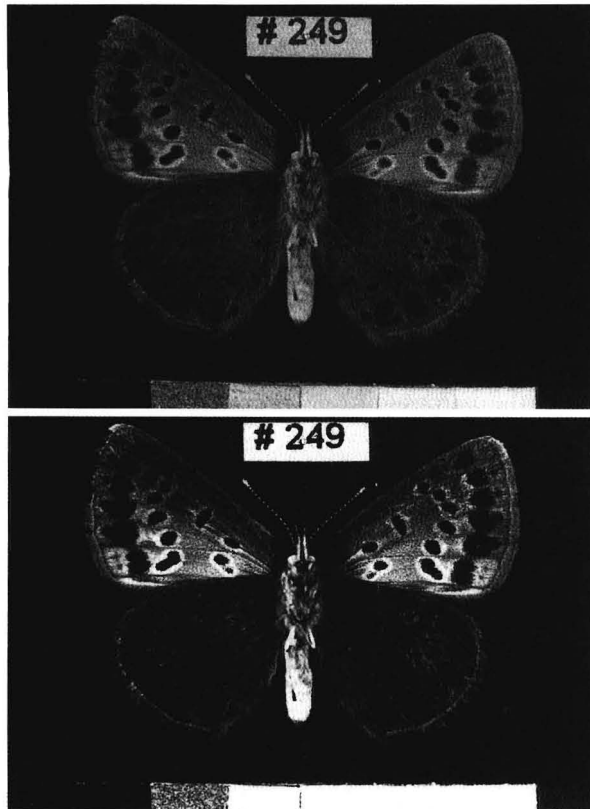


FIG. 3. UV photograph of the underside of one female *Polyommatus icarus* reared on leaves of *Lotus corniculatus*. Both prints were produced from the same negative but on photographic paper of differing grades and accordingly illuminated for different time periods. They illustrate the influence of a minor change in photographic processing. A calibrated gray scale (cf. Fig. 4) included in the photographs allows for a comparison up to a certain degree despite the different appearance of the prints. Width of a step of the gray scale is 5 mm. *Upper print*: Illumination for 5.5 sec, aperture 5.6 on grade 1 paper. *Lower print*: Illumination for 10 sec, aperture 5.6 on grade 5 paper.

visible wing patterns. And there is no reason to presuppose a special function of UV wing patterns as a signal in visual communication.

More elaborate techniques must be applied when studying UV wing patterns as signals used in visual communication. Only when there are very strong differences, without intermediates in UV reflectance, will UV photography be useful in such a context. This might be the case when comparing wing patterns with and without strongly reflecting structural colors. However, even then UV photography will provide rather coarse qualitative results only and individual variability of UV reflectance may be missed (cf. Endler 1990, Brunton & Majerus 1995, this study). Variation in spectral information within the UV range that may be important in communication will also not be apparent with UV photogra-

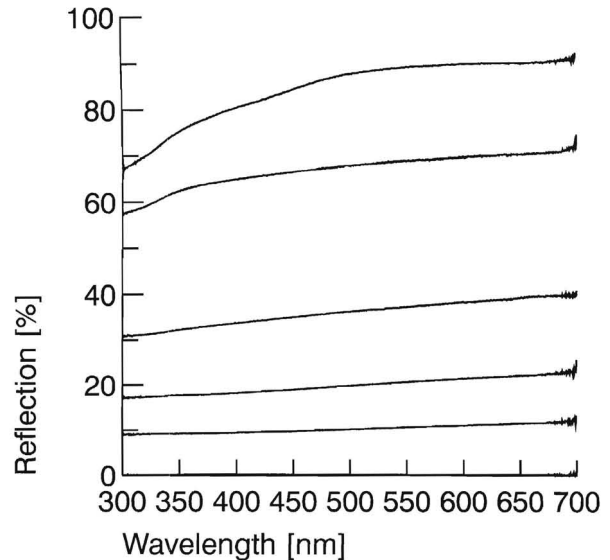


FIG. 4. Spectral reflection of the steps of the gray scale shown in Figs. 1 and 3. The higher the reflectance values the brighter the areas appear in the photographs. The curve for the darkest step almost falls in line with the abscissa. Mean spectral reflection for any given range of wavelengths may easily be obtained from the very flat, almost horizontal curves. The UV range is from 300 nm to 400 nm and the visible range is from 400 nm to 700 nm. *Abscissa*: Wavelength in nanometers. *Ordinate*: Reflection in %, i.e., the amount of light reflected at a given wavelength as compared to a white standard assumed to reflect 100%.

phy. For these reasons, in the study of communication or sexual selection (Bennett et al. 1994b), the method of choice is reflectance spectrophotometry. The whole range of 300 nm to 700 nm needs to be covered by the measurements, that is from the ultraviolet to the red. Comparison of obtained spectra can be done by appropriate statistical procedures (Endler 1990, Cuthill et al. 1999, Knüttel & Fiedler 2001). So far, for only very few species is information available on the physiology of photoreceptors and associated neurons. For these species a physiological model closer to the processes occurring in the organisms may allow to calculate a classification of colors. More details on the assessment of colors in animal communication systems may be found in the excellent works of Endler (1990), Cuthill and Bennett (1993), and Bennett et al. (1994b).

UV photography provides an easy method to assess the spatial distribution of areas of differing UV reflectance. Yet, in the majority of studies qualitative rather than quantitative results were obtained. This means that wing areas were mostly classified as UV-reflecting vs. not UV-reflecting. However, reflectance is a continuous measure that may not easily be assessed in discrete steps (cf. Fig. 2).

Moreover, comparisons between different pho-

tographs and/or studies may be difficult without proper standardization. Brightness and contrast in photographic prints depend on a number of parameters, not all of which might be under the control of the investigator. Important parameters that contribute to variation are amount and spectral composition of illuminating light, spectral transmission of photographic lenses and of UV transmitting filters, the film type, and all kinds of influences on photographic processing including printing during publication. Variation in the appearance of UV photographs may arise from a minor difference in photographic processing as illustrated in Fig. 3. Therefore, a detailed description of optical instrumentation, processing and film material should be given and, as a minimum standard, a gray scale of known UV reflectance should be part of every UV photograph. Such a gray scale will allow comparisons between photographs up to a certain degree because it provides a set of reflectance standards revealing intentional and unintentional differences in brightness or contrast between photographs (Figs. 1 and 3). This method is beautifully described in the pioneering work of Daumer (1958) on the UV patterns of flowers.

UV spectrophotometry yields very accurate quantitative data but requires expensive equipment not available to most systematists and a fair amount of computational data processing. Spectrophotometry is superior whenever spectral information will be required to answer biological questions. However, in contrast to UV photography, spectrophotometry will not provide easily comprehensible spatial pattern information. Hence, for taxonomic purposes where the recognition and documentation of qualitative similarities and discrepancies in wing patterns is usually the most important goal, properly standardized UV photography will continue to be the preferred method, though at the cost of loss of quantitative information.

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