The role of ultraviolet reflectance and pattern in the pollination system of Hypoxis camerooniana (Hypoxidaceae)

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Abstract

Apart from floral morphology and colours perceived by the human eye, ultraviolet (UV) reflectance acts as an important visual advertisement of numerous flowering plant species for pollinators. However, the effect of UV signalling on attracting pollinators of particular plant species is still insufficiently studied, especially in the Afrotropics. Therefore, we studied the pollination system of Hypoxis camerooniana in montane grasslands of Mount Cameroon, West/Central Africa. We focused mainly on the effects of the flowers’ UV reflectance on its visitors. We experimentally removed UV reflection from petals either completely or partially. Thereafter, flower visitors were recorded and pistils were collected post-flowering to quantify germinated pollen tubes per treatments. The most important visitors were bees, followed by flies. Due to their contacts with reproductive organs bees are considered as the primary pollinators. Visitation rates were lower when UV reflectance was completely removed, whereas the decrease of frequency on half-treated flowers did not differ significantly from control treatments. The complete removal of UV also affected bees’ landing behaviour, but not that of flies. We showed that the presence of UV reflectance is more important than UV pattern for bees visiting flowers of H. camerooniana. We hypothesize that exploiting all flowers irrespective of their pattern can be more efficient for pollinators in the open grasslands of high altitudes to spot these relatively scarce flowers by their UV reflectance. Furthermore, we highlight the necessity of both experimental and natural controls in similar studies to control for additional effects of the used UV manipulations.

Keywords: Afromontane grasslands; floral traits; foraging behaviour; Mount Cameroon National Park; pollination interactions; UV manipulation.

Introduction

Unlike humans, many insect pollinators are sensitive to the ultraviolet (UV) part of the electromagnetic light spectrum in addition to the visible spectrum (Briscoe and Chittka 2001). Ultraviolet light is reflected by flowers of ~25 % of angiosperms, with the highest reflectance found in plant species with yellow flowers (Chittka et al. 1994; Papiorek et al. 2016). Consequently,
the UV vision helps floral visitors in recognition of individual flowers of such plants which differ in their UV colouration from other plants in the community (Johnson and Andersson 2002). To increase distinction by certain groups of pollinators, some flowers create a contrasting pattern of UV absorbance and reflectance on the surface of their petals, whereas others contrast petals and reproductive parts by an inverse pattern of absorbance and reflectance of UV light. Floral guides (Penny 1983; Dinkel and Lunau 2001; Lunau 2006; Papiorek et al. 2016) and the so-called bullseye patterns (Lunau 1992a; Koski and Ashman 2014, 2015a, b), which has reflecting apices and absorbing bases of petals, are among the most commonly known examples of this phenomenon. These UV patterns are believed to improve the identification of the landing and/or foraging parts of flowers, or mimic such parts to the pollinator (Lunau et al. 2017). Their importance was shown in numerous studies revealing the influence of UV patterns on pollinator visitation preferences (e.g. Burr et al. 1995; Campbell et al. 2010; Horth et al. 2014; Koski and Ashman 2014; Peterson et al. 2015) and behaviour (Hansen et al. 2012).

The specific colour vision, which includes UV, of some insects and spectral properties of flowers have evolved into mutualistic relationships between plants and their pollinators. One of the best understood systems of vision is that of bees (Peitsch et al. 1999). It has yellow UV-reflecting petals (Fig. 1) and yellow UV-absorbing anthers, consequently creating a contrasting central pattern in the flower. Firstly, we aimed to gain insights in the unknown pollination system of this endemic plant. Secondly, to study the role of UV on the visitation frequency; behaviour and pollination success of H. camerooniana, we used the same approach as Johnson and Andersson (2002) by manipulating flowers with an UV-absorbing cream either by complete removal of the UV reflectance, or by maintaining the UV reflectance on half of petals, i.e. changing the UV pattern (Fig. 1). Our study extends the previous work of, e.g., Johnson and Andersson (2002) by including a natural unmanipulated control to test the influence of the experimental treatments on the flower visitation frequency.

Materials and Methods

Study locality

This study was carried out at the montane grasslands above Mann’s Spring (~2250 m a.s.l.) on Mount Cameroon, the highest mountain in western and central sub-Saharan Africa (4095 m a.s.l.; Cable and Cheek 1998). It is situated in the Southwest region of Cameroon (4.203°N and 9.170°E), offering a wide range of habitats (see Cable and Cheek 1998) and hosting a great biodiversity including endemics with exceptional ecological features (Bergl et al. 2007). Especially ecotones along the timberline, found also at Mann’s Spring, harbour many species which are not found elsewhere on the mountain.

Study plant

The genus Hypoxis contains an estimated 90 species in Africa, North and South America, South-Eastern Asia and Australia, with the centre of diversity in Southern Africa (Singh 1999). These plants are characterized by their bright yellow flowers, lanceolate and densely hairy leaves. They are weak competitors and thus grow mostly in places with low vegetation cover. Hypoxis camerooniana (Hypoxidaceae) is a perennial pyrophytic herb, restricted to high elevations of the Cameroonian Volcanic Line (Cable and Cheek 1998; GBIF Secretariat 2019). Its leaves are tristichous, 50 cm long and 0.5–2 cm wide, covered with golden hairs, recurved and ± prostrate to erect. On a scape up to 25 cm tall 5–7 flowers can be found (African Plant Database 2019).

On Mount Cameroon, we always observed only 1 or 2 active flowers per plant. The flowers have a short lifespan, they open at midday and usually last 1 day or less.

Hypoxis species are used across Africa as traditional medicine and were reported to have a wide spectrum of pharmacological properties (Ncube et al. 2013). Hypoxis hemerocallidea has already
been known for its strong UV reflectance and due to its relatively robust flowers was used for manipulative experiments with UV-absorbing cream (Johnson and Andersson 2002). Outside of the two sweet-smelling species H. fischerii var. zernyi and H. goetzei East African Hypoxis flowers appeared to be without scent (Wiland-Szymańska 2009). So far, no nectar has been found in any Hypoxis species (Johnson and Andersson 2002; Rudall 2002; Ren et al. 2019).

Some studied Hypoxis species are pollinated by solitary bees and honeybees (Singh 1999; Johnson and Andersson 2002), while the autogamous H. decumbens attracts ‘generalist insects, like dipterans’ (Raimúndez and Ramirez 1998). Furthermore, pollen- and tepal-feeding beetles were observed in Southern and Eastern Africa (Steiner 1998; Wiland-Szymańska 2009). No data on pollination and visitation of H. camerooniana exist.

UV manipulation

The study was carried out in October and November 2016. To manipulate the UV patterns on flowers, we followed the approach of Johnson and Andersson (2002). The studied specimens of H. camerooniana were randomly selected in the study area. At daybreak, just after opening of selected buds, four different treatments were applied: (i) UV100% treatment, i.e. complete removal of UV reflectance from the flower using UV cream on all petals of the flower; (ii) FAT100% treatment, i.e. a control for UV100%, all petals were covered with duck preen gland fat, a non-UV-absorbing cream compound; (iii) UV50% treatment, i.e. the UV cream was used on three out of six petals, covering every second petal; and (iv) FAT50% treatment, i.e. a control for UV50%, every second petal was treated with the non-UV-absorbing compound. Both UV cream and non-UV-absorbing cream were applied carefully using cotton swabs. Besides these four treatments, natural (Natural control) non-manipulated flowers were studied to control for the effect of any treatment on flowers. The UV-absorbing chemicals were equal amounts of Parsol 1789® (butyl methoxydibenzoylmethane) and Parsol MCX® (ethylhexyl methoxycinnamate) dissolved in the duck preen gland fat as a solvent (Andersson and Amundsen 1997; Johnson and Andersson 2002). On each day of the experiment, 10 plant specimens were selected in the grasslands and randomly treated, two replicates of each treatment per day, resulting in a total of 50 experimental plants. When two flowers were found on a single experimental plant, we applied the same treatment for both. Each experimental flower was recorded by a security camera (VIVOTEK IB8367-T with IR night vision) for 24 h following Mertens et al. (2018); however, most of the flowers were short-lived and closed at the beginning of the night after ~12 h of recording. Due to the short lifespan of flowers, all their visitors were certainly observed. Afterwards the recordings were watched, and all floral visitors

Figure 1. A flower of H. camerooniana: (A) a normal photograph, (B) a UV photograph of a non-manipulated flower, (C) a UV photograph with half absorbent cream treatment (UV50%), (D) a UV photograph with full absorbent cream (UV100%) treatment.
were noted. Besides arrival of visitors we also identified them to the most detailed taxon level as possible, and we noted their behaviour (both landing behaviour and activity after landing, e.g. feeding on pollen) and touches to reproductive organs, which allows us to better distinguish between visitors and potential pollinators.

After camera removal, stigmas of the recorded flowers were collected and stored in ethyl alcohol. Germinated pollen tubes were stained and counted later in the lab using fluorescence microscopy following the methods described by Dafni et al. (2005) to see how changes in visitation frequency potentially caused by the experimental treatment affect the plant’s pollination success.

To check the floral UV pattern, UV photographs were taken using a Canon EOS 80D DSLR camera with a Helios 44-2 lens; UV conversion (i.e. replacing the internal hot mirror filter by a custom UV band pass filter) was done by LifePixel (Mukilteo, USA). During the picture taking, a 5-W UV flashlight was used for lighting (Fig. 1). To demonstrate the effect of experimental treatment, we measured reflectance of three flowers per treatment type (FAT50 %, Natural control and UV50 %) with 10 repeated measures per flower, using an Ocean Optics (Largo, USA) Jaz spectrometer. The graph depicts the mean of the repeated measures per treatment for the range of 300–700 nm (Fig. 2A). The bee and fly colour visual system was then mapped using the Troje model for flies (Troje 1993; Fig. 2B) and the colour hexagon for bees (Chittka 1992; Fig. 2C).

Statistical analyses
To standardize the sampling effort (differences in flower longevity, as well as in the case of one or two flowers per experimental plant), all visits were transformed to visitation frequencies (no. of visits per hour and flower). This visitation frequency data (no. of visits per hour and flower) did not show normal distribution due to an overabundance of null values. In consequence, we used non-parametrical tests, being a permutational analogue of ANOVA and MANOVA in PRIMER 6 v. 6.1.13 and Permanova+ v. 1.0.3 (Anderson et al. 2008). Post hoc tests were used to compare the frequencies between the different treatments, with the recording day treated as random effect. Similarly, the effects of treatments on insect behaviour were tested by a permutational MANOVA. To check differences in amount of morphospecies and pollen tubes (i.e. the non-frequency data) we tried to implement generalized mixed-effect models, specifically Poisson, quasi-Poisson and zero-inflated distributions. However, due to a combination of the high overabundance of zero values and the negative values of the maximum likelihood estimations of the models, we were not able to apply these parametric methods and therefore, the non-parametrical tests (permutational analogue of ANOVA) were applied as well. The dependency of the number of pollen tubes on visitation frequency was tested by linear regression in STATISTICA (Statsoft, Inc 2011).

Results
Visitors of H. camerooniana
Considering all 50 observed plants, a total of 281 visitors were recorded. During daytime the flowers were mostly visited by bees (192 visits) and flies (59 visits), the only other considerable group of visitors were skipper butterflies (four visits). All other visitors (five visits) were evaluated as accidental and thus merged (Fig. 3). All bee visitors were composed of a single abundant morphospecies of solitary bee (187 visits) and the substantially rarer honeybee (Apis mellifera; five visits). The less abundant flies were considerably more taxonomically diverse, compared to bees, with nine recognized morphospecies. Bees visited the studied flowers mainly during morning hours, whereas fly visitation was distributed throughout the day [see Supporting Information—Figs S2 and S3]. Contrastingly, night visitors were rare (21 visits by 10 morphospecies) and consequently, with much lower visitation frequencies [see Supporting Information—Fig. S1]. Based on contacts with reproductive organs (Fig. 3; Table 1) bees may be considered as the main pollinator.

Effects of UV pattern on visitors
Individual treatments significantly affected visitation frequency during the day (F_{ps} = 6.71; df = 4; P_{perm} < 0.001). UV_{100 %} was significantly lower than all other treatments except UV_{50 %}, which differed from FAT_{50 %} and Natural control. The highest visitation frequency was observed on untreated plants, but these did not significantly differ from the other two control treatments (FAT_{100 %}, FAT_{50 %}; Fig. 4). During the night, there was no significant treatment effect on frequency of flower visitors (F_{ps} = 0.36; df = 4; P_{perm} = 0.851). Visitation frequency was significantly affected by the treatment for both bees (F_{ps} = 6.13; df = 4; P_{perm} < 0.001) and flies (F_{ps} = 3.92; df = 4; P_{perm} = 0.009). In both visiting groups, FAT_{100 %} and FAT_{50 %} treatment has a significantly higher frequency than UV_{100 %} but UV_{50 %} was significantly lower than FAT_{100 %} for flies only (Fig. 4). The non-treated control (Natural control) significantly differed from UV-manipulated plants for bees only (Fig. 4). There was no significant effect of treatment on the number of morphospecies observed on the flowers (F_{ps} = 2.08; df = 4; P_{perm} = 0.103).

Effects of treatments on visitor behaviour
We found a significant effect of treatment on bee landing behaviour (F_{ps} = 5.04; df = 4; P_{perm} = 0.004), but not on fly landing (F_{ps} = 1.08; df = 4; P_{perm} = 0.373). On UV_{100 %}-treated flowers, bees landed mostly on anthers, whereas in other treatments bees usually landed on the petals before moving to anthers and stigma (Fig. 5). When collecting pollen, bees usually touched both anthers and stigmas, whereas flies had considerably fewer contacts with the reproductive organs during their visits (Table 1). There was no significant effect of treatment on bees (F_{ps} = 0.49; df = 4; P_{perm} = 0.770) and flies (F_{ps} = 0.44; df = 4; P_{perm} = 0.950) behaviour after landing. Bees spent 95 % of the flower visit duration by collecting pollen, while flies spent most time (68 %) crawling, sitting and flying between individual floral parts [see Supporting Information—Fig. S4].

Effect of treatment on the plant
The number of germinated pollen tubes significantly differed among treatments (F_{ps} = 3.66; df = 4; P_{perm} = 0.010), mainly due to a significantly higher number of pollen tubes germinated in non-manipulated flowers (Fig. 6). The pollen tube count increases with number of visits by bees (r = 0.57, P < 0.001; Fig. 7), but not of flies (r = −0.0073, P = 0.962).

Discussion
Our study demonstrated that H. camerooniana is mainly pollinated by bees, confirming previous studies on pollination of Hypoxis plants (Singh 1999; Johnson and Andersson 2002),
with the notable exception of H. decumbens, pollinated by flies in Venezuela (Raimúndez and Ramirez 1998). In addition to the previous studies of the genus based on visitation rates, we have confirmed that bees are the most efficient pollinators of H. camerooniana since: (i) bees were the most common visitors; (ii) in a large percentage of visits they are in contact with reproductive organs, which is expectable for these voracious pollen feeders; and (iii) their visits significantly increased numbers of germinated pollen tubes in stigmas. Contrary to Johnson and Andersson’s (2002) observations on H. hemerocallidea in South Africa, there were only few visits of honeybees compared to the abundant visits by a single morphospecies of small solitary bees. Moreover, honeybees seemed to be mostly searching for nectar, although we did not observe any nectar in flowers of H. camerooniana, consistent with other Hypoxis species (Johnson and Andersson 2002; Rudall 2002; Ren et al. 2019).

UV and visitor frequency

The UV signal of flowers influenced the particular visitor frequency in different ways. Bees visited flowers more often when at least half of the petals reflected UV. However, although not significant, even the control flowers treated with non-absorbing cream differed in bee (but not fly) visitation frequencies from untreated flowers. The drop in visitation frequency between the treatments and their respective controls is consistent with the previous study of H. hemerocallidea (Johnson and Andersson 2002; Rudall 2002; Ren et al. 2019).
in which fewer honeybees (A. mellifera) visited flowers with the floral UV reflectance obscured. While in alpine communities of New Zealand, Campbell et al. (2010) found experimentally manipulated flower colour, and not UV reflectance, to be more important for the visitation rates, other studies on UV pollinator visitation preferences showed similar results to ours (Peter and Johnson 2008; Rae and Vamosi 2013; North et al. 2014; Koski and Ashman 2014). Ultraviolet reflectance was revealed as highly important for bees pollinating Eulophia zeyheriana (Orchidaceae; Peter and Johnson 2008), whereas the general visitation rates of various visitors declined after manipulation of UV reflectance in two Rudbeckia species (North et al. 2014), Mimulus guttatus (Phrymaceae; Rae and Vamosi 2013), and Argentina anserine (Rosaceae; Koski and Ashman 2014). Therefore, UV reflectance plays an important role in pollinator attraction, but it can differ among flowering species, since other floral traits, such as scent, shape and colour, could be equally important.

Additionally, we have shown that having at least some UV reflectance is more important for selection of H. camerooniana flowers by bees than its UV pattern (sensu Koski and Ashman 2014), as flowers with fully covered petals by the UV-absorbing cream differed in bee visitation from those with all petals fully reflecting UV. Flies, however, although also showing a cream differed in bee visitation from those with all petals covered with the UV-absorbing cream compound; flies mostly landed directly on the anthers and immediately started to collect pollen, whilst they landed mostly on petals of the flowers that at least partly reflected UV (i.e. all other treatments). This has proven that a disturbance of the UV pattern may change bees’ behaviour. Likewise, other colour patterns, such as floral guides or bullseye patterns, are considered to increase the plants’ reproductive success by helping pollinators to orientate to the flower centre (Waser and Price 1985; Dinkel and Lunau 2001; Leonard and Papa 2011; Papiorek et al. 2016). However, bees actually make their first antennal contact preferably at the UV-absorbing floral area, irrespective of its spatial position within a flower (Papiorek et al. 2016). Therefore, one would expect bee visitors of H. camerooniana to prefer the centre of flowers with the UV-absorbing anthers, which is not the case. We thus hypothesize that in H. camerooniana, the UV-reflecting petals probably act as a landing platform, making flowers more visible for potential pollinators in its typical habitat of burnt montane grasslands, since the general UV reflection of similar grasslands vegetation is low (<5 %; Caldwell et al. 1983).

Methodological biases of UV manipulation

When Johnson and Andersson (2002) used the genus Hypoxis for the experimental manipulation of floral UV reflectance to study the response of insect pollinators, they did not include the experimentally untreated plants (Natural control). They thus did not control for the effect of experimental manipulation on natural insect behaviour. In our experiment, which based the methodology largely on the referred study, we demonstrated that such experimental setting is useful to investigate the effect of floral UV signalling on visitors. But at the same time, we discovered that experimental controls (i.e. flowers covered by the non-UV-absorbing cream compound; FAT100%, FAT50%) can differ from the untreated natural flowers. The experimental controls showed lower (but not statistically significant) visitation rates than the natural control for bees. Furthermore, the numbers of germinated pollen tubes on stigmas of natural control flowers of H. camerooniana were significantly higher compared to all treated flowers, apart from the control with fully covered non-UV-absorbing cream. These lower visitation rates and lower number of germinated pollen tubes could be explained by several factors, e.g. less evaporation of scents or changes in the glossiness of the flower. It also proved that we did not cause pollination during handling of experimental flowers.

Additionally, we showed that this effect can be visitor-specific. Flies, generally a more olfactory-oriented group than bees...
(Roy and Raguso 1997), were not affected by the experimental controls at all. For this group, the UV manipulation treatments (UV100%, UV50%) did not significantly differ from the Natural control.

Figure 4. Effect of UV pattern manipulation on visitation frequencies of _H. camerooniana_ for (A) day; (B) night; (C) bee; and (D) fly visitation during the day separately. Note: scaling of the Y-axis is not standardized due to the substantially lower number of visits between day and night, and between bees and flies. Means (bars) and SE (whiskers) are shown. The same letters above the columns indicate non-significant differences in the pairwise post hoc tests. See Materials and Methods for the description of treatments.

Figure 5. Effect of UV pattern manipulation on bee landing behaviour of _H. camerooniana_ flowers. See Materials and Methods for the description of treatments.

Figure 6. Effect of UV pattern manipulation on the number of germinated pollen tubes in stigmas of _H. camerooniana_. Means (bars) and SE (whiskers) are shown. The same letters above the columns indicate non-significant differences in the pairwise post hoc tests.
are shown. Ultraviolet (UV) reflectance. Means (bars) and SE (whiskers) on flowers of Hypoxis camerooniana after manipulation of their flowers. Hypoxis camerooniana flowers. Similar manipulative studies to control for the substances used in floral manipulation. Consequently, we strongly encourage ‘calibration’ of results by controlling for the chemical vector’s (duck preen gland fat in our case) effects in similar experimental studies. In summary, although the duck preen gland fat is a commonly used vector for the UV-manipulating agents (e.g. Johnson and Andersson 2002; Peter and Johnson 2008; Welsford and Johnson 2012; Rae and Vamosi 2013), it affects natural insect behaviour. Furthermore, the thickness of the cream layer or amount of cream in the treatments has not been considered in our study but might play a role in the attraction of flies. The primary pollinators of H. camerooniana in the Afromontane grasslands of Mount Cameroon were bees. When UV reflectance was completely removed visitation rates of bees decreased, whereas the decrease of frequency on half-treated flowers was not significant (although it decreased as well when considering all daytime visitors). The complete UV reflectance removal changed the landing behaviour of bees as well, confirming that altering the natural UV patterns affects both visitation rates and behaviour. Furthermore, based on our results we also encourage the inclusion of a natural control in the experimental designs of similar manipulative studies to control for the substances used in floral manipulation. The following additional information is available in the online version of this article—

**Figure S1.** Frequency of nocturnal visits of Hypoxis camerooniana flowers.

**Figure S2.** Diurnal changes in visitation frequencies on flowers of Hypoxis camerooniana after manipulation of their ultraviolet (UV) reflectance. Means (bars) and SE (whiskers) are shown.

**Figure S3.** Diurnal changes in fly visitation frequencies on flowers of Hypoxis camerooniana after manipulation of their ultraviolet (UV) reflectance. Means (bars) and SE (whiskers) are shown.

**Figure S4.** Bee (A) and fly (B) behaviour on flowers of Hypoxis camerooniana after manipulation of their ultraviolet (UV) reflectance. There was no significant effect of treatment on both bee ($F_{ps} = 0.49; df = 4; P_{perm} = 0.770$) and fly ($F_{ps} = 0.44; df = 4; P_{perm} = 0.903$) behaviour after landing.

**Data** An excel file with the data used for analyses and graphs is available in the online version of this article.

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**Conflict of Interest** None declared.

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