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Increased solar radiation and soil moisture determine flower colour frequency in a mountain endemic plant population

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Abstract
Flower colour is a fascinating trait that has been of interest to biologists for its utility in understanding variation in natural populations and its role in floral evolution. Here, we investigated whether the co-occurring white and pink flowers of individual plants of the Drakensberg near-endemic taxon, *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt (Hypoxidaceae) are an example of phenotypic plasticity or of flower colour polymorphism and what environmental factors may drive observed changes. We used both field and growth chamber studies to test the relationship between environmental variables and the shift in the proportion of the two flower colours over the flowering season. We found that single flowers do not change colour over time, but some individual plants are potentially responding to changes in environmental conditions by producing pigmented flowers later in the flowering season, which suggests that the trait could be plastic rather than a true polymorphism. The field data showed that soil moisture along with an interaction between ultraviolet (UV) radiation and temperature best explained the change in the number of pigmented flowers over the flowering season but none of our treatments in the growth chambers had a significant effect on the change in the number of pigmented flowers. Given the relationship between anthocyanin production and environmental stress, our field findings suggest that soil moisture plays an important role in facilitating stress tolerance and that *R. baurii* var. *confecta* may produce anthocyanins to prevent tissue damage from increased temperature and UV later in the flowering season.

Keywords Hypoxidaceae · Mountain grassland endemic · Soil moisture · Flower colour variation

Introduction

Plants may exhibit flower colour variation within populations and across species' ranges (Schiestl and Johnson 2013; Van der Niet et al. 2014) and the role of ecological factors in evolutionary consequences of this variable trait (Strauss and Whittall 2006; Pirie et al. 2016; Le Maitre et al. 2019) may explain why this variation is maintained within natural plant populations (Narbona et al. 2018; Sapir et al. 2021). Moreover, the underlying cause of variation in this trait could be a result of phenotypic plasticity or a consequence of a distinct polymorphism. Narbona et al. (2018) defined flower colour polymorphism (FCP) as an instance where distinct, and different coloured, flowers (termed 'morphs') coexist in a single population and are not due to recurring mutations (Ford 1945; Huxley 1955). Globally, many flowering plant genera exhibit colour polymorphism. Irises (Iridaceae) are an emblematic group that contains FCPs worldwide (e.g., *Iris atropurpurea* Baker and *I. haynei* Baker [Lavi and Sapir 2015], *I. brevicaulis* Raf and *I. fulva* Ker-Gawler [Martin et al. 2008], and *I. lutescens* Lam. and *I. pumila* L. [Souto-Vilarós et al. 2017]). FCP has been observed in the Iberian Peninsula endemic plant species, *Silene littorea* Brot. (Caryophyllaceae), where two out of 17 surveyed populations comprised three distinct colour morphs – white, light and dark pink (Casimiro-Soriguer et al. 2016). Occasionally, a more localized scenario of FCP may be apparent. Notably, a single species may comprise varying frequencies of different morphs in populations through time (e.g., *Linanthus parryae* [Gray] Greene; Schemske and Bierzychudek 2001) and

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space (e.g., African genus *Protea* L.; Carlson and Holsinger 2015). It is important to distinguish whether the observed variation represents real FCPs or phenotypic plasticity of flower colour.

Selection pressure exerted by pollinators can facilitate the diversification of floral traits (Fenster et al. 2004; Schiestl and Johnson 2013) as pollinators might target one morph over the other (Schemske and Bradshaw 1999). Pollinator preference (learned, innate or spontaneous) for rare morphs may result in negative-frequency-dependent selection where the frequency of morphs decreases as they become common (e.g., rewardless orchid *Dactylorhiza sambucina* (L.); Gigord et al. 2001) or positive-frequency-dependent selection where the frequency of morphs increases as they become common (e.g., foraging bias of bumblebees *Bombus terrestris* (L.) to conspicuous blue flowers; Smithson and Macnair 1996). In some instances, pollinator preference is not observed – some pollinators show a lack of preference for any colour morph (Imbert et al. 2014), while in other instances, the preference of herbivores for certain morphs may potentially counter flower colour selection exerted by pollinators (e.g., *Raphanus sativus* L.; Irwin et al. 2003). In other systems, observations at study sites have not revealed any pollinators—e.g., *Mimulus verbenaceus* in Vickery (2008) and *M. luteus* in Carvallo and Medel (2010). Such examples indicate that pollinator selection may not always drive the maintenance of flower colour variation relative to other potential selection agents.

Abiotic selection pressures could also lead to variability in flower colour. Of the three major classes of pigments (e.g., carotenoids, anthocyanins and betalains, Grotewold 2006; Tanaka et al. 2008), anthocyanins aid in plant protection from abiotic stressors such as heat and water-deficit environmental conditions (Warren and Mackenzie 2001; Strauss and Whittall 2006; Rausher 2008; Vaidya et al. 2018), which supports a correlation between flower colour morph and environmental variables. Moreover, plants may respond to reduced soil moisture and ultraviolet (UV) stress in their environments (Berardi et al. 2016; Twyford et al. 2018; Koski and Galloway 2020), by inducing the production of anthocyanins that may provide an effective sunblock by absorbing parts of UVA and UVB spectra (Chalker-Scott 1999; Takahashi and Badger 2011). Consequently, fluctuating environmental conditions may lead to shifts in flower colour either within a growing season or over generations due to the potential of pigmented and unpigmented morphs to have a differential ability to tolerate environmental stressors (Dick et al. 2011) and plant fitness may vary for different morphs (Carlson and Holsinger 2010, 2013). However, there may not always be a fitness cost for unpigmented morphs (Twyford et al. 2018). For instance, in *Protea aurea* (Burm.f.) Rourke subsp. *aurea*, white morphs had larger inflorescences and higher seed production compared to pigmented morphs (Carlson and Holsinger 2010, 2013). Identifying environmental variables that underpin flower colour variability within populations may provide an opportunity to better understand colour morph maintenance and subsequent floral evolution.

The South African near-endemic Drakensberg plant genus, *Rhodohypoxis* Nel, (Asparagales: Hypoxidaceae), presents an opportunity to study whether shifts in the number of different flower colours – from mostly white flowers to mostly pink flowers – over the flowering season are due to changes (if any) in environmental variables and to test whether there is a difference in fitness between the individuals with different flower colours. This genus comprises six species, among which the most widespread *Rhodohypoxis* species, *R. baurii* (Baker) Nel, exhibits flower colour variation. *Rhodohypoxis baurii* var. *confecta* Hilliard & Burtt shows flower colour variation within single populations where white and pink flower colours are prevalent at different time points over the flowering season. Originally, Hilliard and Burtt (1978) hypothesized that *R. baurii* var. *confecta* flowers may open white and age through pink to red; however, this remains to be tested. Interestingly, bright, intensely pink, flowers of *R. baurii* var. *confecta* are usually only observed in a handful of individuals near the end of the flowering season. Field observations have not revealed clear pollinators for these plants and crossing experiments have shown that the species has a mixed mating system, with some ability to self-fertilize (Ferreira et al. in prep). Further, previous work in this taxon indicated that an increase in soil moisture corresponded with a shift from mostly white flowers to mostly pink flowers over the flowering season (Gardiner and Glennon 2019). Here, we used field data and a controlled growth chamber experiment to address the following: (1) we established whether the flowers change colour over time by asking if individual plants produce flowers of different colours, or do individual plants maintain a single flower colour through the season, (2) is there a relationship between flower colour, UV, and soil moisture and (3) are there fitness differences (i.e., flower number and seed set) between the two flower colours in the field and under growth chamber conditions?

Materials and methods

Study system

Rhodohypoxis species occur in mountain grasslands above 1650 m a.s.l. (Hilliard and Burtt 1978). Here, we focused on *R. baurii*, identified by the formation of colourful carpets of white and pink flowers in the Drakensberg grasslands (Hilliard and Burtt 1978). These plants are perennial geophytes that die back in January–February

until mid-October when the summer rainfall begins which encourages plants to produce leaves and flowers. Although the distribution of *R. baurii* is mainly in the Drakensberg grasslands located in Lesotho and South Africa, the species ranges between Barberton (Mpumalanga) and Mthata (above 1100 m a.s.l.; Eastern Cape; Hilliard and Burtt 1978). Three *R. baurii* varieties can be distinguished by their flower colour and the ecological characteristics in their distribution (Hilliard and Burtt 1978): *R. baurii* var. *confecta* grows at high altitudes (above 2000 m a.s.l.) on damp, grassy slopes of partly shaded rock flushes or cliff faces, *R. baurii* var. *baurii* Hilliard & Burtt grows in ephemerally damp, rocky grass habitats, whereas *R. baurii* var. *platypetala* Hilliard & Burtt occurs on shallow, dry, stony soils, over rock sheets.

In general, *R. baurii* individuals have more than one flowering event where multiple, independent flowers emerge over the flowering season, and the flowers may be white, pale, or bright pink depending on the *R. baurii* variety. Flowers emerge from buds within two or three days to become fully open, and they usually senesce after 14–20 days. In this study, we focused on *R. baurii* var. *confecta* because populations comprise individuals with flowers that are white, pale pink or bright pink whereas *R. baurii* var. *baurii* flowers are predominantly pink and *R. baurii* var. *platypetala* flowers are predominantly white (Hilliard and Burtt 1978).

We sampled individuals from a population of *R. baurii* var. *confecta* ('taxon' hereafter) that occurs in the northern Drakensberg near the Sentinel Peak car park, Free State, South Africa at 2550 m a.s.l. (Hilliard and Burtt 1978). Our focus on a single population enabled us to control for population-specific environmental or ecological variables, particularly because flower colour variation in this taxon may be population-specific. At the study site, this taxon is widespread both above and below the hiking trail along the ridge and mountain grassland. Further, the plants are exposed to harsh environmental conditions such as relatively low soil moisture (~ 3%), direct sunlight from mid-morning to sunset (~ 9h30–18h00), freezing temperatures and some shading in the early morning due to the surrounding mountains. We sampled within an area that is approximately 7.5 hectares between the car park and up to the zigzag portion of the hiking trail (– 28.7274, 28.8917). All field work for this study was conducted at this site.

Do individual flowers change colour over the flowering season?

During the 2019 flowering season, we tagged 40 *R. baurii* var. *confecta* individuals at the study site. We used labelled tent pegs to identify each plant throughout the flowering season. We then photographed individual flowers from emergence to senescence on all tagged plants (i.e., the full flowering season) every second day from November 11 to December 6. Flowers were photographed next to a paint swatch with different shades of pink to establish a visual baseline in cloud cover/other atmospheric conditions to best distinguish the pale pink and bright pink flower colours (see Fig. 1). We anticipated using the swatches to assess colour as continuous data; however, the varying weather conditions made it difficult to accurately quantify the lighter shades of pink, therefore we elected to score flower colour as a binary character (pigmented/pink = 1 or unpigmented/white = 0). The same scoring system was maintained for each day when the same plants/flowers were photographed. Notably, during our study, only three 'bright' pink flowers were observed and did not live long enough for us to photograph from emergence to senescence (likely due to herbivory). We used a generalized linear model of the binomial family in the base *stats* package in R version 4.2.1 (R Core Team 2022) to assess if an individual's flower colour changed over the flowering season, where flower emergence and senescence were set as a fixed effect. To minimize potential errors, all photos were taken by the same observer, at roughly the same time of day in the morning (~ 08h00), with the same camera. All statistical analyses performed in this study were conducted in R version 4.2.1 (R Core Team 2022).

Monitoring flower colour shift and potential environmental correlates in the field

To establish whether the number of white and pink flowers changed between the beginning and the end of a flowering season, we visited the *R. baurii* var. *confecta* population in the beginning of the flowering season (end October or early November) and towards the end of the flowering season (in December) annually between 2018 and 2021. We haphazardly laid out a one-squared metre quadrat at 12

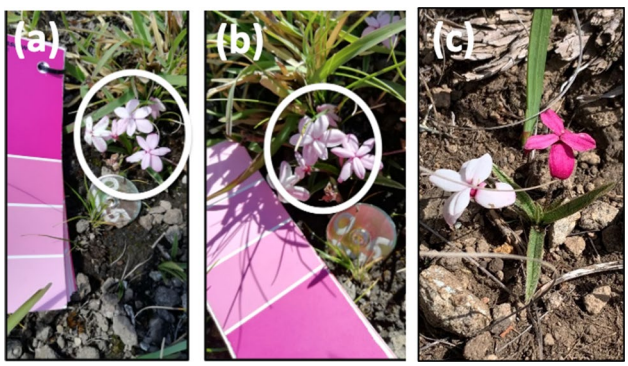


Fig. 1 Comparison of marked *Rhodohypoxis baurii* var. *confecta* individuals at the beginning (**a**) and end of the flowering season (**b**) and an individual plant producing a white flower and later a pink flower (**c**). A colour swatch was photographed next to the flowers to standardize colour measurements

different locations at the study site for each sampling bout where some quadrats were 2 m apart and others were up to 500 m apart. Given that quadrats were dropped haphazardly at different positions within this area, over the different visits, it is unlikely that one individual plant was counted more than once. In each quadrat, we counted the number of pink and white flowers (regardless of whether an individual plant comprised multiple flowers), number and colour of buds, number of senesced flowers and number of developed seed capsules. In late 2018, we collected seeds from each quadrat into an individually marked coin envelope where the flower colour of senesced flowers could be identified. We used flower number and seed set (for seeds collected in 2018) as a measure of fitness between the white and pink colour flowers. Soil moisture data were also collected during the 2019–2021 field trips to compare soil moisture and the number of pink and white flower colours at the start and end of the flowering seasons. While counting the number of flowers within each quadrat, three soil moisture measures were taken at random points within the quadrat using a Hydrosense II metre (Campbell Scientific Inc., Utah, USA) and the volumetric soil water content (VSWC) was averaged to calculate one soil moisture reading per quadrat. Soil moisture data were not collected in 2018. After completing data collection over the 4 years (2018–2021), we used two-sided *t*-tests to assess if there was a difference in the number of pigmented flowers and soil moisture between the beginning and end of each flowering season. Further, we constructed linear mixed-effects models using the *lme4* (v1.1–31; Bates et al. 2015) and *lmerTest* (v3.1–3; Kuznetsova et al. 2017) packages to test if the interaction of year and flowering season influenced the response variables of soil moisture and number of pigmented flowers, with quadrat set as a random effect for each model.

In addition, for the specific dates that we visited the population (between October 2018 and December 2020), we obtained daily data for 12 parameters (see below) from the National Aeronautics and Space Administration (NASA 2021) Langley Research Centre (LaRC) Prediction of Worldwide Energy Resource (POWER) Project funded through the NASA Earth Science/Applied Science Program (https://power.larc.nasa.gov/data-access-viewer/.). These data were extracted using the same GPS coordinate from our study site. We used the following parameters as potential predictors of flower colour shift over the flowering season (presented in alphabetical order): (1) all sky surface UVA irradiance (W/m^2), (2) all sky surface UVB irradiance (W/m^2), (3) all sky surface UV index (dimensionless), (4) all sky surface PAR total (W/m^2), (5) clear sky surface PAR total (W/m^2), (6) dew/frost point at two meters above the surface of the Earth (°C), (7) Julian date, (8) precipitation (mm/day), (9) profile soil moisture (0–1), (10) root zone soil wetness (0–1), (11) surface soil wetness (0–1) and (12) temperature at two meters above the surface of the Earth (°C). We also included year of sampling as an additional parameter. The scale of 0–1 indicates a completely water-free soil at 0 and a completely saturated soil at 1. We constructed a correlation matrix (Supplemental Fig. 1) using the *corrplot* package (v0.92; Wei and Simko 2017) to exclude highly correlated parameters in our analyses using a correlation coefficient cutoff of $r < 0.70$. Consequently, five parameters were selected for further analyses, with year of sampling as an additional parameter: temperature, profile soil moisture, UV index, Julian date, and precipitation. Although we were not able to explore other environmental variables, such as soil pH and soil nutrients, it may be likely that soil pH and soil nutrients facilitate changes in flower colour at a small microhabitat scale over the flowering season, within a single locality. This may be a result of the rains leaching nitrogen, phosphorus and other basic nutrients from the soil as the flowering season progresses. However, we noted that white and pink flowers in this taxon occur in close proximity at a microhabitat scale (sometimes ~ 1 cm apart; see Fig. 1c) in the field, so it seemed unlikely that soil pH could change drastically enough to influence flower colour differences within such a small distance.

We constructed 22 possible explanatory linear models using the base *stats* package in R to test for their contribution in explaining the changes observed in number of pigmented flowers over the flowering seasons. Our global model comprised all selected parameters as predictor variables and the response variable while the null model comprised only the response variable and no predictor variables. Further, we constructed candidate models for each predictor variable and response variable and then included different possible combinations of the predictor variables (see Table 3 for specific details) to compare against our hypothesis that soil moisture and UV likely explain the change in the number of pigmented flowers. We also included an interaction model of year and season of sampling as a possible predictor as we did in the previous section. Log transformation was done on the response variable to improve normality. We used the *AICcmodavg* package (v.2.3–1; Mazerolle 2020) to compare the 22 different model combinations according to their ΔAICc (Akaike information criterion) values where the lowest ΔAICc values indicated the most supported model (Mazerolle 2020).

Environmental variable effect on flower colour emergence

Between 2016 and 2018, a total of 100 mature *R. baurii* var. *confecta* individuals were collected from the study site and the individuals were sampled at least two meters apart. Plants were potted in individual 10 cm pots using a Culterra mix (50% topsoil, 50% compost) and then housed

and maintained in the greenhouse at the University of the Witwatersrand, Johannesburg, South Africa, under similar irrigation and temperature conditions. Soil moisture was monitored on a weekly basis in the greenhouse and then maintained at a VSWC of ~ 20% through automated sprinkler irrigation. At the beginning of the 2018 and 2019 flowering seasons (October), all plants were hand watered with 10 ml of a seaweed emulsion mixture (SeaGrow) diluted in 200 ml of water to induce emergence and growth.

Using 96 plants from the collected stock, we designed a two-way factorial growth chamber experiment to investigate the effect of progressive water-deficit and exposure to UV light conditions on plant growth and flower colour, following a similar experimental design to Twyford et al. (2018). We selected two different soil moisture levels (well-watered and water-deficit) and two different levels of light conditions (UV lights and normal lights), after which four experimental treatments were developed. In the first treatment, plants were exposed to UV light and well-watered conditions, to test for the effect of UV exposure and adequate soil moisture conditions on plant growth and flower colour (UV + /W +). In the second treatment, plants were exposed to a combination of UV exposure and water-deficit conditions to enable a decoupling of both variables from each other (UV + /W–). For a third treatment, plants were not exposed to UV but well-watered as a control for the experiment (UV–/W +). In the fourth treatment, plants were not exposed to UV but experienced water-deficit conditions to test the effect of water deficiency on plant growth (UV–/W–).

Four walk-in growth chambers (CONVIRON; model no PGW40) were used to run the experiment. In each growth chamber, reference carbon dioxide and relative humidity were maintained at 400 ppm and 60%, respectively, while temperature (°C) and light intensity (incandescent and fluorescent light levels) fluctuated to simulate daily changes in the greenhouse environment – i.e., 100% light intensity and 25 °C during the day (6am – 6 pm) and 0% light intensity and 17 °C at night (6 pm–6 am) in each chamber. Prior to the experiment, we downloaded Conviron general trend data from each chamber and then placed iButton data loggers (Fairbridge Technologies) in each growth chamber to record temperature. This enabled us to assess if set-points and actual temperature and light intensity were comparable between the growth chambers. We found that the general trend data and iButton temperature data matched across the growth chambers. A week prior to the start of the experiment, plants were moved from the greenhouse and placed into the growth chambers to allow them to acclimate to set chamber conditions. We used mature plants to remove potentially confounding factors from developmental effects during growth. At the time of collection in the field in 2017, plants had white/very pale pink flowers and no definitive pink flowered plants were collected. These individuals did not have flowers prior to the experiment in 2019, so we were unable to assign plants with known flower colour to specific experimental treatments. Six mature plants were haphazardly assigned to each treatment and each growth chamber contained all four treatments (e.g., treatment 1 was replicated four times); across the four chambers, a total of 24 plants were used per treatment. We rotated each treatment among the four chambers to reduce potential chamber-specific effects.

Normal incandescent (42 W/230 V) and fluorescent lights (54 W/840 CRI and colour temperature) were used in each growth chamber, with an addition of blacklight blue fluorescent lights (FT 36 W/T8 BLB; 1200 nm) only in the UV exposure treatments. We attempted to use a digital UV light metre (UV-340A, Lutron Electronic Enterprise Co., LTD) to quantify the UV light emitted by a combination of regular white lights and blacklight blue lights onto the plants in the UV treatments, however, we were not able to achieve this as the blacklight blue lights had to be turned off before entering the growth chambers to avoid UV exposure. After turning off the blacklight blue lights, we waited at least 30 min before entering the growth chambers to collect data. At the study site, average VSWC is generally 20–30% after rain and ~ 3% when the soil is relatively dry. As experimental water-deficit experiments may be plant and experiment-specific, with the size and type of pot affecting soil moisture retention, we conducted a pilot water-deficit study on potted *R. baurii* var. *confecta* and found that at a VSWC lower than 10%, plants started to wilt and became photosynthetically constrained whereas plants were physiologically active (response to gas exchange measurements) at a VSWC above 10%. A VSWC of 10% was then used as a maximum threshold for the water-deficit treatment. To achieve this VSWC during the experiment, plants in the water-deficit treatment were only watered every four days, while plants in the well-watered treatment were watered daily and received ~ 200 ml of water per day.

The experiment started in the second week of November 2019 and ran for six weeks between November and December 2019. The duration of the experiment corresponded with the natural flowering season of *R. baurii* var. *confecta* in the field. During the experiment, plants were haphazardly rotated between the chambers, within their same experimental treatment, at weekly intervals to minimize chamber/block effect. Soil moisture was measured in the first week of the experiment in the morning (before 09h00) using a Hydrosense II metre with a 12-cm moisture probe (Campbell Scientific Inc., Utah, USA) to confirm VSWC differences in the well-watered and water-deficit treatments. Three soil moisture measurements were taken within each pot, and the VSWC was averaged to obtain one soil moisture reading per pot. Over the course of the experiment, growth chambers and plants were regularly inspected for pests.

During the experiment at weekly intervals, we measured/counted five vegetative and floral traits on all plants to assess the fitness and growth of the plants under each of the treatments over time: number of leaves, length and width of the largest leaf, the number and colour of floral buds and the number and colour of flowers. Measurements of largest leaf length were multiplied by the corresponding leaf width to estimate an approximate measurement of leaf size. After the experiment ended, we constructed linear mixed-effects models using the *lme4* (v1.1–31; Bates et al. 2015) and *lmerTest* (v3.1–3; Kuznetsova et al. 2017) packages to test for the possible effect of the treatments on the traits. Plant ID and week number were included as covariates in the models, with chamber set as a random effect. When a significant effect of the treatments was found, we used Kruskal–Wallis multiple comparison post hoc tests to assess if the traits differed between the treatments.

Table 1 Results from a generalized linear model showing that individuals flowers of *Rhodohypoxis baurii* var. *confecta* on single pedicels do not change colour between the day of emergence and senescence

Source	Estimate	*SE*	z value	*P* value
(Intercept)	1.190e + 00	4.317e-01	2.756	0.006 **
Day of emergence to senescence	− 2.711e-16	6.105e-01	0.000	1.00

Asterisks denote significant differences at $P = 0.001$

Results

Do individual flowers change colour over the flowering season?

There was no significant change in the colour of individual flowers over the flowering season ($P = 1.00$; Table 1). This means that individual flowers maintained the same colour from bud stage until they senesced (see Fig. 1a, b). In total, 40 flowers were photographed and none of them changed colour from emergence until senescence. However, we observed that although individual flowers do not change colour themselves, one plant, out of the 40 we observed, started the flowering season producing a white flower and that same individual produced a pink flower later in the flower season (Fig. 1c). However, given that we only observed 40 individual plants, it could be that more individuals in the population follow this within-plant shift.

Examining colour shifts and potential environmental correlates in the field

Field counts showed that 4737 white flowers and 8898 pink flowers were counted in quadrats at the study site between 2018 and 2021 (Table 2). We found that changes in the number of pigmented flowers over the flowering seasons were explained by both sampling year ($t = 4.55$, df = 84, $P < 0.0001$) and flowering season (early or late; $t = 2.30$, df = 84, $P = 0.02$), and the interaction of sampling year and flowering season ($t = -2.30$, df = 84, $P = 0.02$). There was a significant difference in the number of pigmented flowers between the beginning and end of the 2018 ($t = -3.51$, df = 13.41, $P = 0.004$), 2019 (Wilcoxon test W = 1, $P < 0.0001$) and 2020 flowering seasons ($t = 4.74$,

Table 2 Counts of floral buds, flowers and measures of the percentage of flowers, average number of seeds per plant of pink and white flowers of *Rhodohypoxis baurii* var. *confecta*. Counts of senesced flowers are also included

Flowering season	Year	Buds		Flowers		Total number of flowers	Percentage of flowers of each colour		Seeds		Average seeds per plant		Senesced flowers
		W	P	W	P		W	P	W	P	W	P	
Early	2018	18	213	269	196	465	58	42	–	–	–	–	–
Late	2018	–	–	536	894	1430	37	63	338	454	0.63	0.51	786
Early	2019	32	19	164	109	273	60	40	–	–	–	–	–
Late	2019	41	123	188	1021	1209	16	84	–	–	–	–	605
Early	2020	18	128	1074	2594	3668	29	71	–	–	–	–	–
Late	2020	18	59	992	1093	2085	48	52	–	–	–	–	440
Early	2021	111	198	817	1364	2181	37	63	–	–	–	–	69
Late	2021	63	64	697	1627	2324	30	70	–	–	–	–	393

All measurements were taken in one-squared metre quadrats at two time points over the flowering season (early; Oct/early Nov and late flowering season; late Nov/Dec) each year from 2018 to 2021 at Sentinel Peak car park, Free State, South Africa

df = 14.58, $P = 0.0003$) but no significant difference was found for the 2021 flowering season ($t = -0.91$, df = 20.77, $P = 0.38$). We found that there were inconsistent shifts in flower colour over the surveyed flowering season (Fig. 2).

We found that changes in soil moisture were explained by flowering season ($t = 3.31$, df = 45.49, $P = 0.002$) as well as the interaction of flowering season and sampling year ($t = -3.31$, df = 45.49, $P = 0.002$) but not by sampling year ($t = 1.63$, df = 45.38, $P = 0.11$). Soil moisture significantly differed between the beginning and end of the 2019 ($t = -11.98$, df = 14.01, $P < 0.0001$) and 2021 (Wilcoxon test W = 34, $P = 0.03$) flowering seasons, with the early flowering season having the lowest average soil moisture (2019 VSWC = 7.69%, $SE = 0.47$, 2021 VSWC = 11.72%, $SE = 1.27$) than the late flowering season (2019 VSWC = 23.94%, $SE = 1.27$, 2021 VSWC = 16.38%, $SE = 1.36$). There was no significant difference in soil moisture between the beginning and end of the 2020 flowering season ($t = -1.77$, df = 18.88, $P = 0.09$).

Our candidate model with three predictor variables, soil moisture, UV index, and temperature, was best supported as determined by the lowest ΔAICc value of 0.00 compared to the other models (ΔAICc > 2.27; Table 3). In general, increases in soil moisture, UV and temperature lead to increases in pigmented morphs in the population (Table 3).

Table 3 Selection summary of 22 model combinations based on six parameters: temperature, ultraviolet (UV) radiation index, soil moisture, precipitation, Julian date, year of flowering season, along with interactions between some of the parameters (indicated by an asterisk)

Parameters	K	AICc	ΔAICc
Soil moisture + UV index * Temperature	6	123.97	0.00
Global	7	126.24	2.27
Temperature + Soil moisture + UV index	5	127.08	3.12
Soil moisture + UV index	4	145.32	21.35
Soil moisture	3	155.75	31.79
Soil moisture + Julian date	4	156.04	32.08
Soil moisture + Temperature	4	157.66	33.70
Julian date + Precipitation + Soil moisture	5	157.88	33.91
Precipitation + Soil Moisture	4	158.00	34.03
Temperature + Soil moisture + Precipitation	5	160.00	36.03
Julian date + Temperature	4	161.98	38.02
Sampling year * Flowering season	5	186.48	62.51
Julian date	3	198.43	74.46
Precipitation + Julian date	4	198.54	74.57
UV index + Julian date	4	199.20	75.24
UV index + Temperature	4	208.09	84.12
Temperature + Precipitation	4	208.33	84.36
Temperature	3	211.70	87.74
UV index	3	214.92	90.96
Null	2	215.02	91.05
Precipitation	3	215.81	91.84
UV index + Precipitation	4	216.35	92.39

All models were compared and ranked according to their support for explaining the change in the number of pigmented flowers of *Rhodohypoxis baurii* var. *confecta* over the flowering season. Model selection was based on Akaike information criterion (AIC) where the top model with the lowest ΔAICc value had most support

Seed set

In 2018, we counted 338 seeds from 536 white flowers and 454 seeds from 894 pink flowers towards the end of the flowering season (Table 2). However, there was no significant difference in the number of seeds collected from the white flowers (mean = 9.46, $SE = 0.89$) and pink flowers (mean = 8.45, $SE = 0.74$; $t = 0.87$, df = 85.19, $P = 0.39$).

Environmental variable effect on flower colour emergence

Soil moisture was measurably higher for well-watered treatments (average VSWC = 17.48%, $SE = 1.20$) than water-deficit treatments (average VSWC = 3.44%, $SE = 1.20$) during the experiment. A general trend showed that individuals in the well-watered treatments experienced a decline in VSWC of no less than 10% per day, whereas 21 VSWC measures of 0% were recorded from individuals in water-deficit treatments during the experiment.

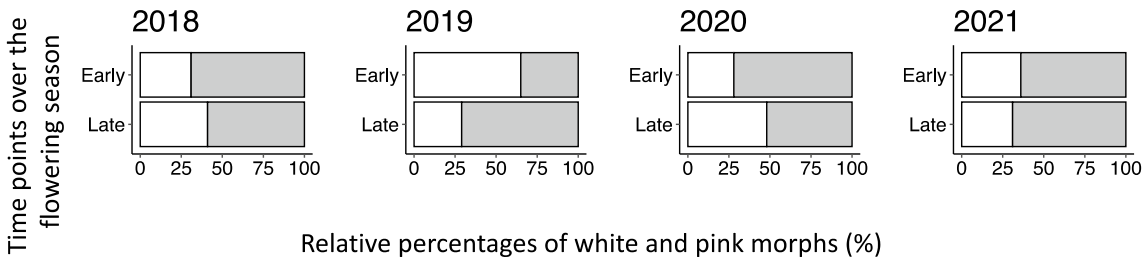


Fig. 2 Relative average percentages of pink (grey bars) and white (white bars) flower colours of *Rhodohypoxis baurii* var. *confecta* measured over different visits at two time points over the flowering season (early flowering season: Oct/early Nov, and late flowering season: Dec) from 2018 to 2021 at Sentinel Peak car park, Free State, South Africa

Individuals in the UV treatments possessed significantly greater largest leaf sizes than individuals in the non-UV treatments (Fig. 3), but all our treatments had a significant effect on largest leaf size (Table 4). In addition, water-deficit treatments had a significant effect on the number of flowers, but no significant difference was found across the treatments (Fig. 3; Table 4).

None of our treatments had a significant effect on the number of pigmented flowers produced during the experiment (Table 4). Further, there was no significant difference

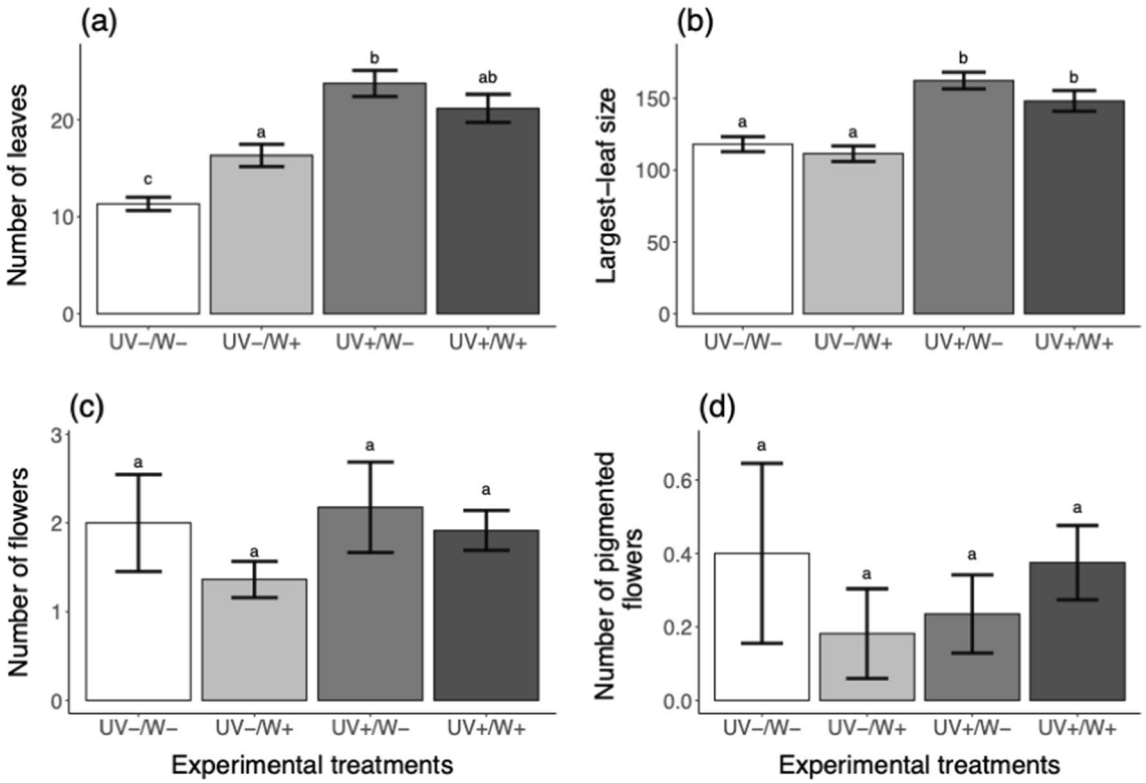


Fig. 3 Vegetative and floral responses of *Rhodohypoxis baurii* var. *confecta* to controlled combinations of soil moisture and UV exposure in a growth chamber experiment under four experimental treatments: UV–/W + = no UV exposure and well watered, UV + /W + = UV exposure and well watered, UV–/W– = no UV exposure and water-deficit and UV + /W– = UV exposure and water-deficit. Traits comprised average values for the number of leaves (**a**), largest leaf size (**b**), number of flowers (**c**) and number of pigmented flowers (**d**). Ninety-six individuals were measured over six weeks. Different letters indicate significant differences between the treatments from Kruskal–Wallis multiple comparison post hoc tests ($P = 0.05$). Errors bars represent standard error

Table 4 Summary of linear mixed-effects models of the vegetative and floral responses of *Rhodohypoxis baurii* var. *confecta* to controlled combinations of soil moisture and UV exposure in a growth chamber experiment under four experimental treatments: UV–/W + = no UV exposure and well watered, UV + /W + = UV exposure and well watered, UV–/W– = no UV exposure and water-deficit and UV + /W– = UV exposure and water-deficit

Treatment	*Number of leaves*				*Largest leaf size*				*Number of flowers*				*Number of pigmented flowers*			
	E	*SE*	*t* value	*P*	*E*	*SE*	*t* value	*P*	*E*	*SE*	*t* value	*P*	*E*	*SE*	*t* value	*P*
UV–/W +	11.00	2.51	4.38	**0.0001**	86.83	12.45	6.98	**0.0001**	1.26	0.86	1.46	0.15	− 0.16	0.28	− 0.56	0.58
UV + /W +	16.18	2.65	6.11	**0.0001**	119.30	13.13	9.09	**0.0001**	1.47	0.89	1.66	0.10	0.10	0.28	0.36	0.72
UV–/W–	5.01	2.45	2.05	**0.04**	89.02	12.15	7.33	**0.0001**	1.89	1.03	1.84	**0.07**	0.04	0.33	0.11	0.92
UV + /W–	18.36	2.58	7.13	**0.0001**	132.37	12.77	10.37	**0.0001**	2.04	0.81	2.51	**0.02**	− 0.09	0.26	− 0.35	0.73
Plant ID	− 0.05	0.02	− 2.05	**0.04**	0.41	0.12	3.42	**0.001**	0.01	0.01	1.07	0.29	− 0.001	0.003	− 0.47	0.64
Week number	2.21	0.46	4.81	**0.0001**	1.44	2.24	0.64	0.52	− 0.06	0.16	− 0.36	0.72	0.08	0.05	1.62	0.11

Plant ID and week number were included as covariates, with chamber as a random effect. Standard error (*SE*) and estimate of regression coefficients (*E*) are reported. Significant *P* values are highlighted in bold where 0.0001 represents values that are too small to report. A significant *P* value means the treatment has a measurable effect on the response variable. The relevant response variable is listed in the top row

in the number of pigmented flowers across the treatments (Fig. 3). No seeds were observed on individuals or collected during the experiment.

Discussion

In this study, we investigated whether individual flowers of *Rhodohypoxis baurii* var. *confecta* change colour over the flowering season. We found that the individual flowers themselves do not change colour throughout the flowering season, but for some individual plants, pink flowers emerge later in the flowering season. Further, we found that there were inconsistent shifts in flower colour over the flowering season across multiple years. For instance, in the 2021 flowering season, there was not a significant change in the number of pigmented flowers between the beginning and end of the flowering season. Together, these data suggest that flower colour may be a plastic trait in *R. baurii* var. c*onfecta* that is due to environmental conditions, which could change annually. Second, we used a field study and growth chamber experiment to test if environmental variables explained the change in the number of pigmented flowers and if environmental variables affected flower colour emergence. Our field data indicated that a combination of soil moisture with an interaction of UV and temperature best explained the change in the number of pigmented flowers. This finding coincides with our expectations because towards the end of the flowering season at the study site, although not entirely consistent across the study years, there are on average more pigmented flowers after a rainy flowering season, and when the sun angle corresponds to high light conditions and more UV exposure.

According to the field component of our study, an increase in soil moisture along with an interaction between UV and temperature explained the increase in pigmented flowers at the study site. Collectively, these three variables are likely to lead to potentially stressful conditions for this species, and anthocyanins enhance a plant's ability to respond to harsh environmental conditions (Warren and Mackenzie 2001; Schemske and Bierzychudek 2001; Strauss and Whittall 2006; Rausher 2008; Koski et al. 2020). The ability to mitigate higher soil moisture, which could induce water clog and bulb rot, is likely important to the plants' survival and reproduction over the flowering season, as *R. baurii* var. *confecta* plants are geophytes and occur on grassy rock slopes above 2600 m a.s.l. It is also likely that pigmented flowers may be more capable of surviving increased UV and temperature than white flowers, especially in the presence of increased soil moisture, where these variables can be associated with the higher altitude of this population (Berardi et al. 2016). Notably, anthers are not exposed in these flowers, so the prospect of UV damage is likely reduced at the high altitude. Recent work has demonstrated that globally floral pigmentation has increased in response to increased UV, and this may be relevant to this high-altitude taxon that experiences higher UV at higher altitude, as it has been noted that many of the higher altitude species of this genus are pigmented (pink) rather than white, like the lower altitude sister species. Flower colour differences are often associated with plant fitness (Strauss and Whittall 2006; Arista et al. 2013), although fitness effects (direct or indirect) can be complicated (Forsman 2016). Here, we found that the difference in the number of seeds between the white and pink flowers was not significant. The nonsignificant trend of pink flowers having a higher total number of seeds (454 seeds) compared to white flowers (338 seeds) could be because there were mostly pink flowers in the population at that time of the flowering season. No seeds were observed on individuals or collected in the field during the 2019 flowering season, potentially because fruiting might have shifted later in the year than expected due to low rainfall prior to the flowering season. Given that these flowers have hidden reproductive structures and possess a mixed mating system, it is unlikely that pollinators would influence the fitness of the different morphs. We initially expected that the pigmented (pink) flowers would have higher fitness because previous work suggested that flavonoids (a type of anthocyanin) reduce heat stress, which in turn improves fertilization success (Coberly and Rausher 2008), or that the pigmented flowers might attract pollinators. However, there were no pollinators observed on the flowers and the mixed mating system with an ability to self supports the similarity in fitness of the colour morphs.

Although unlikely, we cannot exclude the possibility that flower colour variation in this taxon is also due to biotic factors as pollinators might preferentially visit one flower colour at different times over the flowering season. However, preliminary observations at the study site have not revealed any pollinators for *R. baurii* var. *confecta,* and artificial pollination does not induce flower colour change (M.P.M, pers. obs). There were some signs of herbivory on the flowers in the field, but this was not limited to one flower colour over another. Further, although no seeds were observed or collected from the experiment, we do not have data to support that this is because pollinators were excluded from the growth chambers, especially since this taxon is capable of outcrossing and selfing (Ferreira et al. in prep).

Our focus on a single population allowed us to remove potential confounding effects from different populations. However, it is possible that our results may be population-specific and an outcome of adaptation to environmental conditions encountered at the study site. Using a greenhouse experiment where plants were exposed to similar conditions of environmental variables, we aimed to remove factors related to environmental variation and pre-adaptation

before running our experiment. The plants we used as our experimental units did not have flowers prior to the experiment, and we would not have been able to confidently assign known flower colours to specific treatments. However, plants that were collected in the field had flowers that were primarily white or very light pink. As such, not confirming the plant's initial flower colour may have influenced the outcomes of the experiment. Further, in our experiment, individual plants consistently produced the same flower colour morph throughout the experiment. One would expect that more or all flowers in the UV treatments would be pigmented, but the data showed that there was no clear effect of the variables on the flower colour. It is possible that the UV exposure necessary to cause pigmentation in flowers is beyond what we could recreate in a growth chamber and that we did not fully capture the complexity of soil moisture in the growth experiment. This might also explain why there were no bright pink flowers emerging in the experiment.

Conclusion

This study showed that individual flowers of *R. baurii* var. *confecta* do not change colour over time. We also found evidence to suggest that individual plants may produce different coloured flowers at different points in the flowering season, which suggests that these plants are plastic for flower colour and not an example of flower colour polymorphism as defined by Narbona et al. (2018). Further, the interaction of temperature and UV, along with an increase in soil moisture was found to best explain the increase in the percentage of pigmented flowers in the field, which suggests that these plants are likely responding to the changing climate variables in the Drakensberg mountains within a growing season. Future work is necessary to further investigate the strength of soil moisture and solar radiation necessary to stimulate flower colour plasticity in this study system.

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Author contributions KLG and SS conceived the study and design. All authors performed data collection. MPM conducted data analyses. The first draft of the manuscript was co-written by MPM and KLG, and all authors commented on the manuscript. All authors read and approved the final manuscript.

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Data availability Data available at https://osf.io/vzr3b/?view_only=a519260335aa4a3d96fcef4bca627a79

Code availability The R code is available at https://osf.io/vzr3b/?view_only=a519260335aa4a3d96fcef4bca627a79

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval Not applicable.

Consent to participate Not applicable.

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