



No Indication of Background Color Matching in a Population of the Brown-Green Polymorphic Admirable Grasshopper (*Syrbula admirabilis*)

Iván de la Hera · Michael S. Reichert

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Abstract In color polymorphic insects, individuals may track available habitats and select those that better match their color phenotype. This so-called habitat choice mechanism can balance the chances of survival between morphs, equalizing fitness, which can contribute to the long-term maintenance of color polymorphism in a population. We used a population of the brown-green polymorphic admirable grasshopper (*Syrbula admirabilis*) to test whether color morphs differ in their preferences when exposed individually to two contrasting background colors (brown vs. green) in an experimental arena. We obtained data on how the locations (background choices) and frass (number of pellets, as a proxy for the relative use of each color area) of each grasshopper were distributed between the brown and green sections to estimate their background color preferences. We did not detect differences in background color preferences between color morphs in either (molting or non-molting) nymphs or (unmanipulated or color-manipulated) imagoes, rejecting the occurrence of habitat-choice mechanisms. We attribute this lack of habitat selectivity to the widespread presence of a disruptive body

pattern on the back of nymphs and imagoes that blurs individuals' shape and may provide similar crypsis on any background environment. As an alternative hypothesis, we suggest that color polymorphism might be maintained because it impairs prey detection by predators that form search images when hunting, which would reduce overall predation rates in admirable grasshopper populations.

Keywords Color polyphenism · ecdysis · frass pellet · metamorphosis · phenotype-environment correlation.

Introduction

The different variants of a polymorphic species can show a non-random spatial distribution in heterogeneous landscapes, which would lead to phenotype-environment correlations (Sultan 2015; Fokkema et al. 2021). Animal external coloration is one of the most conspicuous traits that might differ among habitats, whereby each color morph has been hypothesized to be more frequent in the habitat where it has higher crypsis, a pattern known as homochromy (Dearn 1990; Fuzeau-Braesch 1972). Homochromy typically results from three different, non-exclusive, mechanisms: (1) selective predation of the less mimetic (maladapted) morphs (i.e. selective disappearance mechanism; Cook et al. 2012), (2) cue-mediated developmental adjustments of the external coloration

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I. de la Hera (✉) · M. S. Reichert
Department of Integrative Biology, Oklahoma State University, Stillwater, OK, USA
e-mail: delaheraivan@gmail.com

to the foreseeable color background conditions of the habitat (i.e. color polyphenism; Simpson et al. 2011; Umbers et al. 2014; Edelaar et al. 2017) and/or (3) behavioral responses that allow individuals to select habitats that better match their color phenotype (i.e. habitat choice mechanism; Edelaar et al. 2019). The latter mechanism can be mediated by imprinting (Davis 2019) or genetic preferences (Jaenike and Holt 1991), which do not necessarily require an individual to assess its crypsis in different environments. Alternatively, under performance-based matching habitat choice, individuals assess the background relative to their own coloration during habitat selection (Camacho et al. 2020). We still have limited knowledge of the relative contribution of all these different mechanisms to explain observed patterns of homochromy, although this information could help us to better understand the evolution and maintenance of color polymorphism in natural populations (Bond 2007). For example, a habitat choice mechanism might contribute to the maintenance of color polymorphisms by minimizing the fitness differences between morphs (Heinze et al. 2021).

Habitat choice mechanisms do not necessarily occur along the entire lifetime of individuals. Natural selection could instead have favored the occurrence of these mechanisms only during specific periods of their life cycle, when the cost of a (color) phenotype-environment mismatch is potentially higher (i.e. partial matching habitat choice hypothesis; Camacho and Hendry 2020). For example, insects show contrasting life-history stages (e.g., larva, nymph, imago) that are associated with different escape abilities and vulnerability to predation (Schultz 1981). These factors could affect the cost-benefit balance for the development of habitat choice mechanisms and, ultimately, shape when they are more likely to be expressed (Merilaita et al. 1999). A critical moment in insects' lives with regard to predation risk is metamorphosis. Unlike insects with direct development (i.e. ametabolous insects), holometabolous and hemimetabolous insects undergo a dramatic reduction in mobility during metamorphosis, so that choosing a cryptic location to undergo this process is expected to increase survival and fitness (Hadjoudj et al. 2014). Despite these apparent benefits, whether insects have preferences for cryptic locations during this period of high vulnerability has been rarely tested (Lucas et al. 2000; Grof-Tisza et al. 2015).

Many species of the order Orthoptera are color polymorphic (Dearn 1990). Grasshoppers also provide several paradigmatic examples of color-dependent habitat choice mechanisms (e.g. Gillis 1982; Karpestam et al. 2012; Camacho et al. 2020). However, although the brown-green polymorphism is very widespread among orthopterans (Schielzeth 2021), green morphs were lacking or very rare in most of the species studied so far (Gillis 1982; Schielzeth 2021, but see Heinze et al. 2021), so it is unclear how common these habitat choice mechanisms are in brown-green polymorphic grasshopper species. Moreover, the contrasting characteristics between their flightless nymphs and their flying imagoes (Schowalter 2016) make grasshoppers suitable for testing whether habitat choice mechanisms are more likely at the more vulnerable nymph stage than at the imago stage, which would support the partial matching habitat choice hypothesis (Camacho and Hendry 2020).

The admirable grasshopper (*Syrbula admirabilis*) is a brown-green polymorphic species for which homochromy has been described in natural populations (Otte and Williams 1972), although the mechanisms underlying this variation are still poorly understood. Color polyphenism had been argued as a potential driver of phenotype-environment correlations in this species but, if it actually occurs in some populations (Otte and Williams 1972), it is not definitively a widespread phenomenon (De la Hera and Reichert 2021). In this study, we used an admirable grasshopper population, for which the existence of dietary-mediated color polyphenism was ruled out in a previous work (De la Hera and Reichert 2021), to explore experimentally whether admirable grasshoppers, either nymphs or imagoes, show color morph-dependent habitat-choice mechanisms when exposed in individual containers to two contrasting background colors. The grasslands in our study site are relatively homogeneous in color and maintain a greenish appearance during virtually all the period of activity of the species (July–October). However, the predominant green grass patches are interspersed at a microhabitat scale with dry grass and bare clay soil, which might create an ideal scenario for brown and green morphs to behave differently and track the color patches that better match their phenotypes. Our study tried to answer this question in an experimental setting with both nymphs and imagoes, and was complemented with two additional experiments. First, we

tested whether color morphs differ in their color preferences during molt (or ecdysis, i.e. incomplete metamorphosis); and, second, we performed an experiment where we manipulated the original coloration of a group of imagoes using acrylic paint in order to explore the existence of processes of habitat matching by self-assessment of color.

Methods

Study Animals and Color Phenotype Determination

The admirable grasshopper is a grassland-inhabiting species that is widely distributed throughout the eastern half of the United States and all of Mexico (Otte 1981). Nymphs of both sexes show brown-green color dimorphism, and five instar stages have been suggested for this species before becoming imagoes. Female imagoes can also be green or brown, with some of them exhibiting intermediate colors (Otte 1981; Otte and Williams 1972). In contrast, male imagoes are brown when sexually mature, although a few males can retain green traces upon their final ecdysis that normally disappear after a few days (De la Hera and Reichert 2021).

Admirable grasshoppers, both nymphs and imagoes, were collected from a field (coordinates: 36°11'03"N 97°10'21"W) located near Lake McMurry, Payne County, Oklahoma (USA). Nymphs were sampled on the 31st of July and 6th of August 2021, whereas imagoes were collected on the 23rd and 25th of August 2021. We characterized the color of nymphs by placing them into visually assigned color categories (green or brown). Nymphs could readily be assigned into one or the other of these categories. Imagoes, however, exhibited more continuous variation in color, so for analyses of imago coloration we used hue values based on photographs. We obtained pictures of the right side of their body against a color standard chart using a camera (Sony DSC-HX50) mounted on a tripod. Pictures were processed using the ImageJ program (version 1.53e, Schneider et al. 2012) in order to obtain the dominant color (hue, measured in degrees) in the pronotum for each individual (see De la Hera and Reichert 2021, for further details on hue calculations). We also measured the hue of the red color standard that was in the background of each photograph. The average hue of the

red color standard was 27.25° (N = 169; 92 nymph and 77 imago pictures), with this measure varying slightly between pictures (i.e. mean minus individual red hue deviation, was between -1.55° and 1.89°). To account for this between-individual variation in photograph conditions, we standardized the pronotum hue values by summing the value of this individual deviation to the original pronotum hue. Although our method for color determination does not account for some factors that are important in classifying color from photographs (Stevens et al. 2007), this approach has been shown useful to quantitatively assess color variation in this same admirable grasshopper population (see details in De la Hera and Reichert 2021). The validity of this approach was supported by the strong association between visual color category assignments and the standardized pronotum hue values (Fig. S1).

Experimental Setting

During the experiments, grasshoppers were housed in individual containers (transparent plastic hinged containers: inner dimensions L29.5 x W10.6 x H6.25 cm), that had been spatially divided in three similar-sized sections with contrasting background colors (L10 x W10.6 x H6.25 cm). Thus, one terminal third was painted in brown (Apple Barrel® 22489E Nutmeg Brown) and the other terminal third in green (Apple Barrel® 20,523 Kelly Green) in order to create two contrasting background color environments at each end of the containers (see picture of experimental set up in Fig. S2). For this purpose, we painted the whole inner surface of the lower tray of the containers (L10 x W10.6 x H2.5 cm), but only the most distal lateral side of the upper tray (W10.6 x H3.75 cm) to allow the entrance of light through the unpainted areas (i.e. longitudinal lateral sides of the upper tray and roof; Fig. S2). The ground (i.e. lower tray) of the central third of the container (approx. L9.5 x W10.6 x H2.5 cm) between the two colored sections was made white by sticking white duct tape on the outer part of the lower tray only. The center of this white area was used as the feeding area, where food (freshly-cut and soaked grass) was provided daily inside the bottom half of a 60 mm diameter petri dish. The cover of this petri dish was also present inside the container, but virtually all the time stuck to the roof via a loop of fishing line glued to the cover and accessible from

outside the container through two small holes (see more details for the purpose of this petri dish cover below).

In order to provide nymphs with suitable molting substrates that differed in their background color (brown vs. green; Fig. S2 and S3), we also hung two straps (approximately 7×2 cm) of synthetic black mesh inside each container. One strap was placed on each end side, running along the most distal wall, from the upper corner to the floor of the container. This black mesh was fixed to the distal end of the roof using a small piece of transparent tape.

We used two long outdoor tables (5 m of overall length and 0.8 m of width) oriented in a north-south direction to place the containers in two parallel columns during the experiments (Fig. S2). Containers were placed on an east-west direction, changing the orientation 180° every day (see below). The whole setup was covered by two 3×3 m Instant Slant Leg Canopies (Ozark Trail©) that prevented the direct incidence of sunlight, which might influence grasshopper behavior and positioning inside the containers (Lactin and Johnson 1997; Harris et al. 2015). We used two pieces of rope that were passed over the center of the containers (crossing the fishing line loop) and firmly tied to the legs of the tables to prevent these light weight containers from being blown away by the wind (Fig. S2).

Daily Maintenance of Grasshoppers During the Experiments

The maintenance protocol during the experiments did not differ between nymphs and imagoes. Thus, day to day work started at around noon and consisted in capturing each grasshopper participating in the experiment, which was temporarily kept inside a 50-ml tube while its experimental container was cleaned (i.e. the frass and some remnants of vegetal matter were removed using an air dust blower) and the old grass in the petri dish was replaced. Following this task, the grasshopper was enclosed inside the petri dish using its cover, the plastic container closed, container's orientation switched 180° (i.e. containers with the green end eastwards in the previous day had this green side now westwards and vice versa), and the grasshopper kept inside the covered petri dish for a few minutes (normally between 5 and 10 min). After this time period, the petri dish cover was lifted by pulling

from the attached fishing line without opening the whole container, allowing the grasshopper to move freely again inside the container. The petri dish cover remained fixed to the roof because of the pressure of the fishing line curvature, so the grass in the dish was always available for the grasshopper. We waited at least one hour after the last container was cleaned to start collecting background color preference data.

Estimation of Grasshopper Color Preferences

Color preference data collection consisted in recording whether grasshoppers were in the brown, green or white section of the container (hereafter background choices), although observations of individuals in the white section were not included in subsequent analyses because this was where the food dish was placed and so individual occurrence in this section was likely related to foraging rather than crypsis. An individual on the transparent side or, very rarely, on the roof of the green or brown end of the container was assigned to the green or brown section, respectively. If an individual was present just on the border between color sections, it was assigned to the area occupied by a larger proportion of its body, or wherever its head was located if the body proportions in each section were similar. The first background choice every day was recorded between 14:20 and 16:00, and another 4 or 5 more choices were recorded before dusk, with the condition that at least one hour elapsed between consecutive observations. An additional last background choice was obtained the following morning (between 9:40 and 12:00) before the corresponding cleaning started and the daily cycle completed.

As a complementary measure of background color preferences, we also counted the daily number of pellets (frass) in the green and brown section of the container, assuming that the preferred background color would have a higher number of pellets. Pellets were counted after approximately 24 h, just before the cleaning of the container each day. Some grasshopper species are known to kick their pellets away (Tanaka and Kasuya 2011), which might alter their distribution within the container. However, this kicking behavior was not common in our grasshoppers. Thus, out of the 49 events where defecation could be video-recorded, in only 6 cases grasshoppers attempted to kick the pellet. However in two of these cases the pellet was not actually hit and, when it was hit, it did not

travel further than twice the length of the grasshopper. We therefore do not think this kicking behavior compromises the usefulness of pellet distributions to infer the background color preferences of admirable grasshoppers within the containers.

Finally, we also recorded when and on which section (brown, green or white) nymphs molted their cuticle (ecdysis). When the molt was not directly witnessed, we indirectly assigned the molting location to the section within the container where their exuvia was found. Some nymphs that had not yet molted on the last day of background color preference data collection, the 14th of August (see below), were maintained in captivity until they molted (last ecdysis date occurred on the 1st of September 2021). During this extended period, we did not collect background choice and pellet count data.

Schedule and Organization of Nymph and Imago Experiments

The captivity experiments designed to obtain background color preference data for nymphs were performed between the 4th and the 14th (11 days) of August 2021. Some nymphs that died or molted during the experiment were replaced by new ones in order to obtain additional independent data. Note that nymph external coloration can be modified after molt and, consequently, could result in changes in their background color preferences (Otte and Williams 1972; De la Hera and Reichert 2021). For this reason, we did not consider background choices of nymphs after molting, regardless of whether this molt led to the emergence of another nymph or an imago (final ecdysis). Background choices obtained on the day that the nymph molted were also excluded to avoid overlapping information with the molting site selection data (see above). This means that the number of days each nymph participated in the experiment differed depending on when they died or molted for the first time within the container. Likewise, the instar stage at which each nymph was collected and monitored within the experiment also varied between individuals, but we do not include instar stage as a factor in the analyses because this trait could not always be reliably determined.

Captivity experiments for imagoes were performed between the 23rd of August and the 2nd of September 2021. First, imagoes stayed four days in

the containers, where maintenance work and background color preference information were collected as described above. This initial 4-day period was used to characterize the natural background preferences of imagoes (control phase) inside the containers before color manipulation. During the cleaning after the fourth day of data collection, and before they were released back into the container again, all imagoes had their pronotum and head (avoiding the eyes, antennae, chewing structures and palpi) painted either green or brown (exactly the same paint used for the containers); the color applied was selected randomly, and regardless of their original coloration. This color manipulation is known to influence background preferences in other grasshopper species (Gillis 1982; Karpestam et al. 2012; Wennersten et al. 2012). Background choices and pellet count data were then recorded during a maximum of four more days (experimental phase).

Statistical Analyses

We recorded background color preference data for 86 nymphs, but we only considered individuals for which we had observations of at least five background choices and five pellets in total, after summing those occurring in the brown and green section. Note that observations from the white section were excluded from all analyses (see above). For the 50 nymphs with sufficient data, we noted that the difference in the overall number of choices in the brown section minus the overall number of choices in the green section (hereafter, between-section difference in background choices) was positively correlated with the same difference for the number of pellets (i.e. number of pellets in the brown section minus number of pellets in the green section; hereafter between-section difference in pellets; see Results). According to this, we entered these two variables (between-section differences in background choices and pellets) into a Principal Component Analysis (PCA), where the first principal component (PC1) reflects the relative preference of each nymph for each background color in the container. Thus, a nymph with positive PC1 values had comparatively more background choices and pellets in the brown section than a nymph with negative values, whose background choices and pellets were relatively more frequent in the green section. In order to test whether nymph color affected the background

color preferences inside the containers, we performed a general linear model where PC1 values were analyzed in relation to the visually-determined color category of the nymphs (brown vs. green). We used this two-level factor instead of the quantitative measure of hue of the pronotum because hue values for nymphs were clustered in two easily distinctive, and contrasting, groups (Fig. 1 and S1).

For a subsample of 36 nymphs that had five or more background choices and five or more pellets (excluding those on the white section) for each of the two possible container orientations (i.e. whether the green section was oriented to the East or to the West) during the experiment, we estimated the repeatability of the PC1 values between container orientations. This was done to assess the extent to which our background color preference estimates (PC1) were consistent between individuals, which may indicate true preferences rather than chance occurrence on one side or the other. Repeatability estimates were obtained using the R library *rptR*, where PC1 values (two values per individual: one for each container orientation, i.e. green section to the East or to the West) were

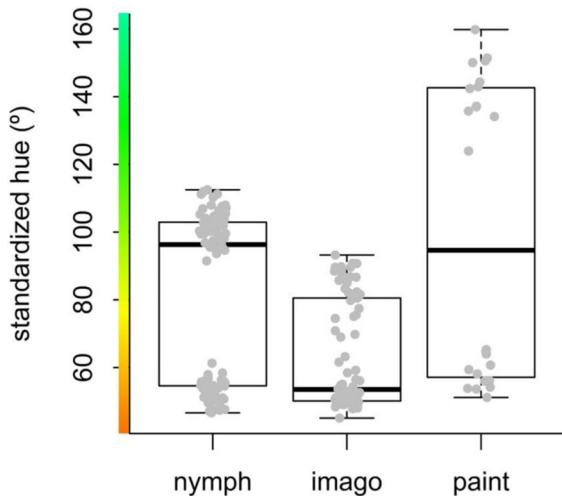


Fig. 1 Variation in the standardized hue of the pronotum (obtained from photo analysis against a red color standard, see Methods) between nymphs, imagoes and the acrylic paints (brown and green obtained from pictures of 12 containers) used in this study. Grey dots show all individual values. Box-plot shows medians (horizontal thick lines within the boxes), percentiles 25–75 (boxes), and minimum and maximum values (vertical segments). Note the clear separation of brown and green hue values for nymphs and paint, and the more continuous nature of hue variation in imagoes

included in the model as the dependent variable and nymph identity as a random factor (Nakagawa and Schielzeth 2010).

We used 45 nymphs that molted within the containers between the 4th of August and the 1st of September 2021 to test whether the color section (brown or green) where their ecdysis took place differed between brown ($N=20$) and green morphs ($N=25$). We performed these analyses using G tests implemented in the *DescTools* package (Signorell et al. 2020).

We obtained background color preference data for 77 imagoes (44 males and 33 females). Out of these, 66 (36 males and 30 females) had five or more background choices and pellets before color manipulation (control phase). Similarly to nymphs, between-section differences in background choices and pellets were strongly correlated with each other (see Results), so we also performed a PCA to extract the first principal component (PC1) as a single variable describing the imago background color preferences. Imagoes were less variable in color (hue) than nymphs, and there were many individuals that were intermediate between green and brown in color (Fig. 1 and S1). Because of the uncertainty in the color assignment of some individuals (see above), we decided to use the photograph-based standardized hues of their pronotum as a covariate in the statistical analyses, instead of a categorical color classification. Accordingly, we performed a general linear model that analyzed whether the derived PC1 scores (as surrogates of imago background preferences during the control phase) varied in relation to the standardized hues of the imagoes.

For the imago color manipulation experiment, 21 individuals (8 males and 13 females) provided five or more background choices and five or more pellets, during both the control and experimental phase (see above). These data were used in an analysis that explored the effects of color manipulation on imago background color preferences using the *lme4* package (version 1.1–23; Bates et al. 2015) to fit a general linear mixed model, and the *lmerTest* package (version 3.1-3) to calculate the degrees of freedom of the denominator, F-tests and P values through the Satterthwaite and Kenward-Roger method (Kuznetsova et al. 2017). Thus, the PC1 score for each individual during each phase of the experiment (i.e. control vs. experimental) was included as the dependent

variable; color manipulation treatment (brown vs. green), phase of the experiment (control or experimental phase), and their interaction were used as fixed effects factors; and imago identity was included as a random effect. If imagoes self-assess their coloration and use this information to adjust their background color preferences, we predict a significant interaction between color manipulation treatment and experimental phase. Thus, in relation to the background color preferences in the control phase, any individual (regardless of its original coloration) experimentally painted with green would increase its preference for green (i.e. lower PC1 scores; note that both green and brown imagoes had lower hues than the green paint, Fig. 1), whereas individuals painted with brown would increase their preference for brown if the imagoes were originally green (higher PC1 scores) or would keep it stable if their original color was brown (since brown imagoes and brown paint showed similar hues).

All statistical analyses were done using R version 3.6.3 (R Core Team, 2020).

Results

Color Variation of Background Paint and Experimental Grasshoppers

As previously described for the admirable grasshopper (see Methods), nymphs showed two very distinctive clusters in relation to the standardized hue variation in their pronotum (Fig. 1), with all the individuals in the high-hue cluster being unequivocally identified as green morphs according to visual assignments, whereas all the nymphs in the low-hue cluster were visually identified as brown (Fig. S1). The standardized hues for imagoes were also distributed in approximately two clusters, although the difference between them was smaller, and most imagoes with high hues fell visually in between the two color categories. Thus, they were mainly assigned as intermediate morphs (Fig. S1). The green and brown paint, that was photographed for 12 containers, also showed markedly different standardized hues (estimate = 84.4 ± 3.1 , $t = 27.2$, $P < 0.001$; Fig. 1). The standardized hues of the brown paint overlapped largely with the hues of brown nymphs and imagoes (Fig. 1), whereas the green paint

showed higher standardized hues than all nymphs and imagoes. In any case, the standardized hues of green nymphs was slightly closer on average to the mean hue of the green paint (mean degrees of deviation = $40.41 \pm 0.67SE$) than to the brown paint (mean degrees of deviation = $44.00 \pm 0.67SE$). In the case of the imagoes, green ($N = 1$) or intermediate individuals ($N = 30$) had standardized hues that were always much closer to the brown paint mean (deviation range = 1.1 – 35.1°) than to the green paint mean (deviation range = 49.2 – 83.3° ; Fig. 1).

Background Color Preferences in Nymphs

The total number of background choices (sum of choices for the brown plus the green section) that nymphs ($N = 50$) made was on average 26.2 (range: 7–54), whereas we counted 47 pellets, on average, for each nymph (range: 6–111). The between-section difference in background choices was positively correlated with the between-section difference in pellets (Spearman's $r = 0.67$, $P < 0.001$, $N = 50$; Fig. 2A). These two variables loaded equally when combined in a PCA (variable loadings for the first principal component = 0.71, eigenvalue = 1.60, variance explained = 0.80). There was no evidence that nymph color was related to choice of background: the derived PC1 values did not differ between green and brown nymphs (nymph color effects: estimate = 0.17 ± 0.36 , $t = 0.46$, $P = 0.647$; Fig. 2B).

For the 36 nymphs with enough background choice and pellet data for each of the two possible orientations of the container, the repeatability of the PC1 score (variable loadings = 0.71, eigenvalue = 1.24, variance explained = 0.77) was not different from zero (95% confidence intervals = 0–0.30).

Although slightly more green nymphs performed their molt in the green section of the container, and more brown nymphs molted in the brown section (Table 1), there were not significant differences between green and brown morphs in the background color in which ecdysis took place (G-test = 1.31, $P = 0.519$). These results did not change qualitatively if the individuals that molted in the white section (2 nymph color morphs \times 3 background color options) were also considered in the test (G-test = 1.50, $P = 0.472$).

Fig. 2 (Upper panel) Relationship of the between-section difference in background choices (Y axis) with the between-section difference in pellets (X axis). (Lower Panel) Variation between brown and green nymphs in background color preferences within the experimental containers. Color preferences were estimated from a PCA that included the two variables represented in the axes of the upper panel. Boxplot as in Fig. 1

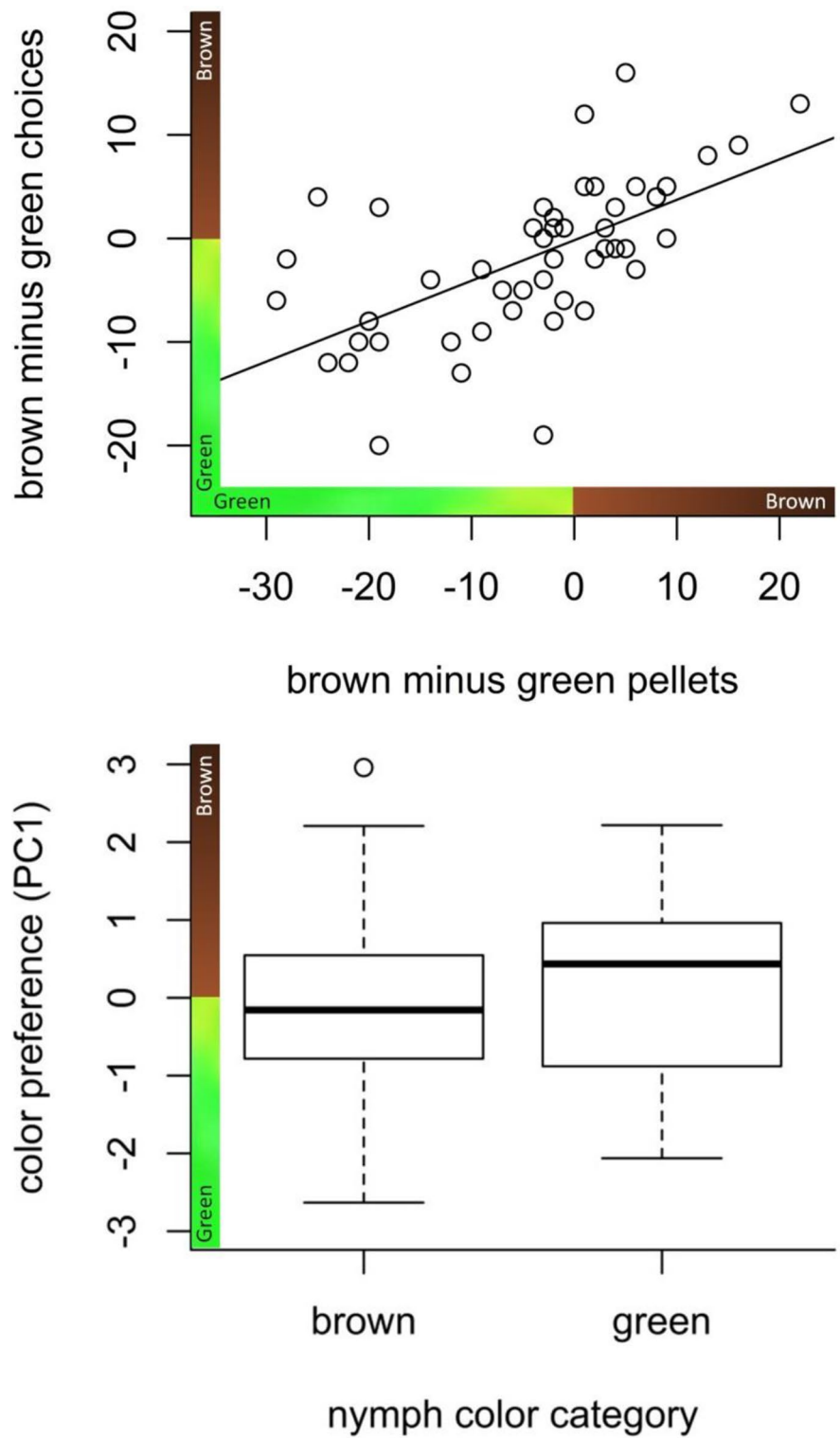


Table 1 Number of brown and green nymphs that molted in each of the three color sections (brown, white and green) of the container

		Nymph color	
		Brown	Green
Container section	Brown	10	8
	White	3	5
	Green	7	12

Background Color Preferences in Imagoes

The total number of choices and pellets obtained during the control phase for the 66 imagoes considered was, on average, 12.1 (range: 5–23) and 36.7 (range: 7–79), respectively. In imagoes, the between-section difference in background choices was significantly correlated with the between-section difference in pellets (Spearman's $r=0.50$, $P<0.001$, $N=66$). The background color preferences (PC1) obtained from a PCA that included both variables (variable loadings=0.71; eigenvalue=1.53; variance explained=0.77) were not correlated with the standardized hue of the imagoes' pronotum before the manipulation (estimate = $-0.015 \pm 0.009SE$, $t = -1.58$, $P=0.118$; Fig. 3A).

Color preferences (PC1; variable loadings=0.71; eigenvalue=1.44; variance explained=0.72) in the 21 imagoes with sufficient data from both the control period with their natural color (mean number of choices and pellets was 13 [range: 8–18] and 46.3 [range: 7–70], respectively) and the experimental period after they were painted (mean number of choices and pellets was 13 [range: 7–22] and 29.9 [range: 11–54], respectively) were not significantly affected by the phase of the experiment in which the data were collected (estimate = $-0.89 \pm 0.48SE$, $t=1.85$, $P=0.081$) or by the color with which they were painted (estimate = $0.40 \pm 0.52SE$, $t=0.77$, $P=0.446$). The interaction between these two effects was also non-significant (estimate = $-0.40 \pm 0.70SE$, $t= -0.57$, $P=0.574$; Fig. 3B).

Discussion

The results of these experiments did not provide evidence for the occurrence of color morph-dependent

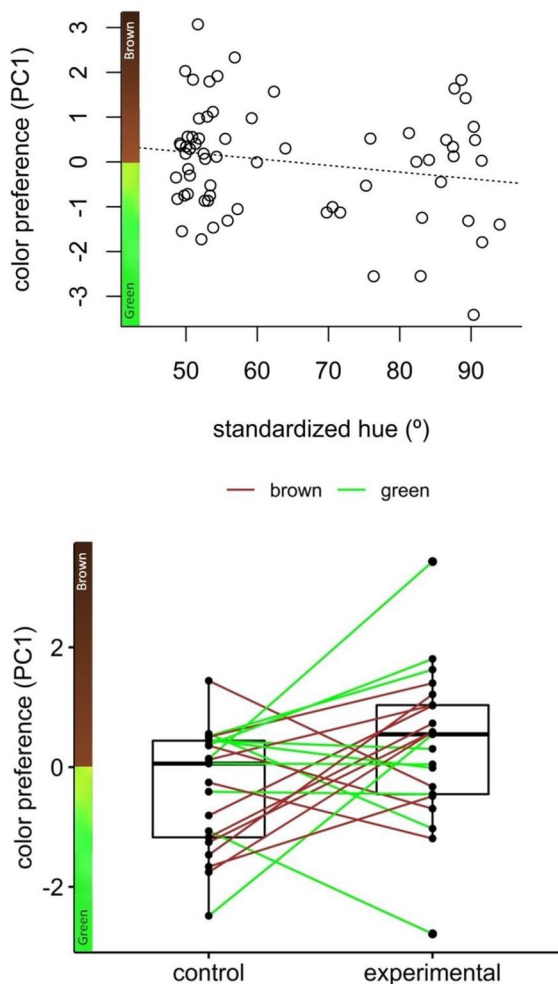


Fig. 3 (Upper panel) Relationship between imago color preferences and standardized hues of the pronotum (estimated from pictures) before the color manipulation (control phase). Color preferences were estimated from a PCA as described in Fig. 2. Regression line (dashed line) is depicted, although it was not statistically significant. (Lower panel) Variation in color preferences before (control phase) and after the color manipulation of the imagoes (experimental phase). Lines connect the color preferences of the same individuals before and after the color manipulation. Brown and green lines indicate individuals that were painted brown and green, respectively. Boxplot as in Fig. 1

habitat choice mechanisms in our admirable grasshopper population. This was true both for nymphs and imagoes, as well as for molting individuals, for whom vulnerability would be the highest, and a habitat choice mechanism potentially most beneficial (see Merilaita et al. 1999; Camacho and Hendry 2020). Background color preferences were also

not significantly affected by the experimental color manipulation, which is further evidence against color-dependent habitat choice, and specifically against the possibility that imagoes assess their own color when searching for a suitable background.

There are some caveats to our experimental design that may affect interpretation of these results and could be addressed in future studies. First, our experimental setting could have been too simple and unnatural, since fully homogeneous green or brown backgrounds, such as those we used to assay color preferences in this experiment, do not occur in natural grassland habitats. In addition, we used canopies to block direct sunlight and equalize as much as possible the distribution of light and temperature in the experimental arenas, but admirable grasshoppers inhabit grasslands where direct sunlight might have a significant influence on their behavior and habitat choices. Another potential limitation of our study was revealed by the hue analyses through photographs, which suggest that the green paint we used as background color was not an ideal color match for the green morphs. This requires a cautious interpretation of the results of the analysis that associated background color preferences (PC1 values) with standardized hue in non-manipulated imagoes (control phase), because the hue of the greenest imagoes was in fact slightly closer to the hue of the brown paint (Fig. 1). Thus, the green imagoes were intermediate between the paint colors and may have been expected to show no background preference, although the brown imagoes were a good match for the brown paint and would have been expected to more strongly prefer the brown background. Accordingly, whether imagoes show color-morph dependent background color preferences should be reassessed using a more suitable green background color. However, this limitation should not have been a major issue in the nymph experiments because here the hues of the green and brown nymphs were closer to the hue of the green and brown paint, respectively, than to the other paint (Fig. 1). This should have elicited different color preferences between green and brown nymphs, if they color-match with the background. Likewise, results from our experiment to assess the existence of self-assessment processes (performance-based matching habitat choice; Camacho et al. 2020) should not have been affected by the

degree of matching between grasshopper and background, because we experimentally manipulated the coloration of the imagoes using exactly the same paint used on the containers, and followed color manipulation procedures that had been successful in similar studies (Gillis 1982; Karpestam et al. 2012).

In general, therefore, our experimental tests supported the null hypothesis of no difference in the background color preferences between color morphs. This interpretation of no color selectivity was reinforced by the lack of repeatability in the background color preferences for a subsample of nymphs that had choice and pellet data under the two different container orientations. This result indicates that there was no within-individual consistency in the background color preferences, and that nymph occurrence on the brown or green section within a container was most likely random. Our results contrast with those from other studies showing experimental evidence for habitat choice mechanisms (Gillis 1982; Ahnesjö and Forsman 2006; Karpestam et al. 2012; Wennersten et al. 2012; Edelaar et al. 2017; Camacho et al. 2020), although these species normally do not have a well-defined brown-green polymorphism (i.e. *Circotettix rabula*, *Sphingonotus azurescens*, *Tetrix subulata* and *Tetrix undulata*). Interestingly, for the only brown-green polymorphic species studied so far, Heinze et al. (2021) found that only the uniformly green morphs color-matched with their background, whereas brown and partially green morphs did not. At this point, it is important to note that admirable grasshoppers lack uniformly green morphs. In contrast, all individuals share some phenotypic characteristics, such as a brown patch in the back of the pronotum accompanied by a black and white longitudinal striped pattern that typically progresses toward the tip of the head (Fig. S4). In other grasshopper species, it has been suggested that morphs with disruptive back body patterns like this have better crypsis across different types of backgrounds, but lower habitat selectivity, than conspecifics without it (Ahnesjö and Forsman 2006; Heinze et al. 2021). In this context, behavioral responses, such as habitat choice mechanisms, might not provide an additional selective advantage for crypsis and would be unlikely to evolve, or be maintained, in the population. This could be the case of the admirable grasshopper, where this pattern in the back of the body is widespread in both nymphs and imagoes and could partly

explain the complete absence of background color preferences in this species.

When compared to previous studies that explored differences between color morphs in grasshopper habitat selection, our experimental approach had some logistical particularities that might be useful to consider in future research. First, we monitored nymphs and imagoes housed in individual containers, which allowed us to reduce the potential effects of social interactions on grasshopper behavior. Note that most previous research was done with grasshoppers housed in communal cages (Karpestam et al. 2012; Wennersten et al. 2012) or studied in natural conditions (Edelaar et al. 2019; Camacho et al. 2020), where the influence of conspecifics and other biotic factors could not be controlled for (but see Ahnesjö and Forsman 2006). Second, we used pellet distribution within the container as an indirect measure of microhabitat selection. The distribution of pellets between the brown and green sections (between-section difference in pellets) was moderately correlated with the distribution of grasshopper background choices (between-section difference in background choices; Fig. 2A), which supports the validity of pellets to estimate background color preferences. Accordingly, we encourage the recording of pellets in the different sections of an experimental arena as a technique that is not particularly time-consuming (pellets can be counted once a day), but allows obtaining a more comprehensive view of habitat preferences than using only sporadic observations of individual habitat choices. This approach can be particularly appropriate in species that rarely or never kick their pellets away (Tanaka and Kasuya 2011), as was the case here. Lastly, since our plastic containers lacked a rough vertical surface from which nymphs could hang safely during ecdysis, the inclusion of mesh straps or another suitable substrate in experimental containers is advisable to facilitate this key developmental process. We observed that 31 of 45 nymphs molted hanging from one of the two mesh straps provided.

Homochromy is a common phenomenon in many insect species (Rowell 1972; Pener 1991) and it has been also described for the admirable grasshopper (Otte and Williams 1972). Homochromy could arise from habitat choice or diet-mediated color polyphenism, but our results, together with another recent study (De la Hera and Reichert 2021), rule

out these mechanisms as drivers of homochromy in our admirable grasshopper population. Consequently, if homochromy actually exists, then, selective disappearance/predation processes would be the main candidate mechanism that could explain this pattern. Accordingly, future studies should focus on this hypothesis, but bearing in mind that the apparently reduced color variation between grasslands in our study area could make homochromy occur at a microhabitat scale in this population (see Otte and Williams 1972).

On a final note, regardless of the potential differences in how each color morph performs in different habitat types, color polymorphism itself could be advantageous, at both the individual and population level, because it can reduce overall predation rates by impairing prey detection by predators that form search images when hunting (Karpestam et al. 2016). Thus, if predators use different clues to detect each color morph (search image formation; Bond 2007), this can eventually lead predators to feed preferentially upon the most abundant phenotype (apostatic selection; Bond and Kamil 1998). This frequency-dependent selection process might be occurring in admirable grasshopper populations and could alternatively explain the long-term maintenance of color polymorphism in this species (Van Leeuwen and Jansen 2010), a possibility that deserves further research.

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Authors' Contribution Both authors designed the study and collected the specimens in the field. IDH performed the experiment and wrote a first draft of the manuscript, on which MSR provided insightful suggestions for improvement of the scope, analysis and wording of the manuscript. Both authors read and approved the final version.

Data Availability Data can be downloaded from this link in FigShare: <https://doi.org/10.6084/m9.figshare.19161959>.

Declarations

Conflict of Interest Authors declare no conflict of interest.

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