Sky radiance and spectral gradient are orienting cues for the sandhopper *Talitrus saltator* (Crustacea, Amphipoda).

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Keywords

Sky radiance, Spectral gradient, Orientation, Sandhopper, *Talitrus saltator*

Summary statement

Sandhoppers were tested in the laboratory under artificial radiance or spectral gradients

The light radiance gradient is used as a compass cue only at certain intensity levels

The spectral gradient is used as a chronometric compass cue

Abstract

The sandhopper *Talitrus saltator* relies on both the sun and the moon compasses to return to the belt of damp sand of the beach in which it lives buried during the day. In addition to the sun, the gradient of radiance and the spectral distribution across the sky could provide directional information that *T. saltator* can potentially use to orient itself during the day even when the sun is not visible (e.g. cloudy sky). The scope of this work was 1) to determine the intensity levels of sky radiance that the sandhoppers use in their zonal recovery and 2) to investigate whether this species relies on the celestial spectral gradient in its zonal recovery. Sandhoppers were tested in the laboratory under artificial radiance or spectral gradients.

Our results show that under an artificial sky, simulating the natural radiance gradient on a cloudless day, sandhoppers orientated toward the correct seaward direction of their home beach, however, individuals lost their ability to use the intensity gradient as an orientation cue when the radiance was attenuated by at least 40%. Sandhoppers were also able to head in the correct seaward direction of their home beach at any time of the day by using the spectral gradient as their only source of visual orientation reference.

Introduction

The supralittoral sandhopper *Talitrus saltator* (Montagu, 1808) is one of the best-known arthropods for behavioral studies on celestial orientation. To return to the belt of damp sand by the sea where it lives buried during the day, this species can rely on both the sun and the moon as cues for compass orientation (Papi and Pardi, 1953; Pardi and Papi, 1953; Pardi and Ercolini, 1986; Ugolini et al., 1999a, b). However, the sun and moon are not the only celestial cues used by this species. Beside astronomical cues, sandhoppers can use local cues, such as the slope of the substratum and the vision of the landscape as references to return to their belt of damp sand (Ercolini and Scapini, 1974; Craig, 1973; Hartwick, 1976, Ugolini et al., 1986, 2006; Ugolini, 2014; Ugolini and Ciofini, 2016). In addition to the sun, sunlight scattering on atmospherical particles generates additional skylight cues, such as a polarization pattern as well as profiles of radiance and spectral distributions (e.g. see Rossel and Wehner, 1984; Wehner 1989, 1997; see also Horváth and Varjù, 2004; Cronin et al., 2014 for reviews). The pattern of skylight polarization provides directional information concordant with that provided by the sun, and helps many arthropods ~~to~~ to orient itself during the day even when the sun is not directly visible (e.g. under cloudy sky conditions) (e.g. see Waterman, 1981; Horváth and Varjù, 2004; Cronin et al., 2014; for reviews). Although the celestial polarization pattern servers as a reliable orienting reference for many arthropods, the sandhoppers do not seem to use it as a compass cue in their zonal recovery (Ugolini et al., 2013).

Only *T. saltator* and the dung beetle *Scarabaeus lamarcki* are known to be able to obtain compass information from the skylight radiance gradient (Ugolini et al., 2009, 2012; el Jundi et al., 2014a). Even though these two species face different ecological problems they both are under the pressure to orientate in the most efficient way: *T. saltator* must return to the damp belt of sand as quickly as possible by a kind of chronometric orientation defined as unidirectional not-vector orientation to avoid death due to dehydration. In contrast, *S. lamarcki* needs to maintain a straight route while rolling its ball away from a dung pile in a randomly-chosen compass direction to avoid intraspecific competition (e.g. see Dacke et al., 2013, 2014; Khaldy et al., 2019).

Although the use of the skylight radiance gradient as an orientation cue was first described in various species of animals, *T. saltator* included, by Verheijen (1978), since then only a few investigations have been conducted to study its role for orientation in arthropods (Wehner, 1997; Ugolini et al., 2009; el Jundi et al., 2014a). In insects, the relevance of the skylight radiance as a compass reference seems to be relatively low and used only in combination with other cues. For instance, the orienting system of the desert ant *Cataglyphis bicolor* is disrupted when allowed to orientate using only this cue (individuals lose their ability to maintain a correct homeward direction but perform phototactic responses toward the solar hemidome instead, Wehner, 1997). The dung

beetle *S. lamarki* (likely not performing time-compensated orientation) can orientate with high precision only by using intensity information in combination with additional skylight cues, such as the skylight polarization (el Jundi et al., 2014a).

Although sandhoppers use the skylight radiance gradient for orientation (Ugolini et al., 2009), it has never been tested how the degree of cloudiness of the sky affects the ability of sandhoppers to rely on this cue for orientation. An additional celestial cue used by some insects to achieve directional information is the spectral gradient. It is characterized by the predominance of longer (460-700 nm) wavelengths in the celestial hemicycle including the sun ("solar" hemidome) and shorter (293-460 nm) wavelengths in the opposite, "antisolar" hemidome (Coemans et al. 1994). Some arthropods, like the honey bee *A. mellifera* (Brines and Gould, 1979; Edrich et al., 1979; Rossel and Whener, 1984), the desert ant *C. bicolor* (Wehner, 1997) and the dung beetle *S. lamarcki* (el Jundi et al., 2015b, 2016), are known to be able to discriminate between the sun and the antisun direction based on spectral information and to use them for spatial orientation. Interestingly, it has been demonstrated (Ugolini et al., 2010) that *T. saltator* perceives UV-blue (390-450 nm) and green (500-550 nm) wavelength of lights, which opens up the possibility that these animals may also derive compass information from the spectral pattern of the sky. The aim of this work was therefore to evaluate the ability of *T. saltator* 1) to detect and use skylight radiance profiles attenuated with respect to that occurring in conditions of clear sky and 2) to investigate the use of the celestial spectral gradient in its zonal recovery.

Material and methods

Adult individuals of *T. saltator* were collected in spring-summer 2013-2017 on a sandy beach in the Natural Park of Migliarino, San Rossore, Massaciuccoli (Pisa, Italy, 43°40'03" N, 10°20'29" E, sea-land axis = 265°-85°). Tests were conducted within 2 weeks after collection. In the laboratory, animals were kept in Plexiglas boxes containing wet sand at room temperature (25°C), under a light:dark (L:D) cycle of 12:12 h in phase with the natural photoperiod. Food (universal dried food for fish, SERA® Vipan Heinsberg, Germany) was constantly available.

We tested groups of 10-12 individuals within one trial, as it has been shown that the directional choice of individual sandhoppers is not influenced by the heading directions of their conspecifics (Scapini et al., 1981). At the end of the experiments, the sandhoppers were released at the collection site.

Artificial radiance gradients

We performed experiments to test the use of radiance gradients and spectral gradients releasing sandhoppers under artificial light conditions. To increase the motivation to rapidly orientate

seaward, the animals were dehydrated for 2-3 minutes before the experiments. The sandhoppers were released at the centre of a transparent Plexiglas bowl (diameter = 18 cm) placed on a horizontal transparent plate (diameter = 30 cm) at local noon (Fig. 1A). The sandhoppers can jump or walk, slipping, trying to climb the wall of the bowl. A single direction for each radially-oriented individual (with the head pointed toward the outside of the bowl and the longitudinal axis of the body oriented no more than ± 45° from the radius of the bowl) was recorded after 2 minutes from each release from freeze-framed images taken by a camera placed below the bowl. Sandhoppers belonging to each group were released only once. A goniometer was set below the bowl to measure the directions taken by the individuals with respect to 0°=North. The bowl was surrounded by an opaline Plexiglas cylinder (diameter = 30 cm) closed at the top by an opaline Plexiglas plate (Fig. 1A). The artificial sky was projected (once reflected by a mirror) onto the circular opaline Plexiglas plate placed over the bowl, using a video-projector provided of a 200w halogen lamp (mod. LC4341, Philips, Made in Austria).

The artificial radiance gradients were characterized through radiometric measurements performed at intervals of 5° along the solar meridian (zenithal distance of the sun = 45°). The spectroradiometer (mod. AvaSpec-2048, Avantes BV, Apeldoorn, Netherlands) was mounted on a stand and placed in the position where the animals started their orientation behavior during the experiments. The spectral radiance (W/m^{2}sr nm) and spectral patterns were measured for both the real sky and our artificial environment (Fig. 1C, D). The radiance perceived by *T. saltator,* P_r, was calculated by weighting the recorded measurements, R(λ) by the spectral sensitivity measured by electroretinogram (ERG) of the sandhoppers, S(λ) (Ugolini et al 2010):

$$P_r = \int_{300\,nm}^{650\,nm} R(\lambda) \cdot S(\lambda) d\lambda$$

The same method was used to evaluate the amount of light perceived by UV-blue and green photoreceptors (Ugolini et al. 2010) substituting green sensitivity G(λ) or blue sensitivity B(λ) of the absorption curves of each visual pigment calculated of each peak measured by ERG to S(λ) in the formula above. The images simulating the natural skylight radiance gradient under a clear sky (Fig. 2A) and with lower radiance gradients (Fig. 2D, G) were designed using the software Matlab® (MATrix LABoratory). The images (Fig.2D, G) were obtained by assigning a grey value to each numerical value of light radiance measured along the solar meridian under natural conditions.

In addition, we designed images attenuated to the gradient present in natural conditions of clear sky by reducing light radiance variations along the solar meridian. In particular, we elaborated patterns attenuated by 40% (Fig. 2D, E) and 75% (Fig. 2G, H).

Artificial spectral gradient

Sandhoppers were released in a confined environment using the device described elsewhere (for a more detailed description see Ugolini et al. 1998, 2005). A white opaline Plexiglas dome (diameter = 80 cm) was placed on top of the bowl. The internal surface of the dome (the artificial sky) was illuminated by a fibre-optic illuminator (Schott KL1500). The end of the light guide (diameter = 8 mm) was accurately placed in a tube at the centre of the bowl (Fig. 1B) which allowed to present a wide stimulus across the inner surface of the dome (dome illumination). The end of a second light guide (diametre 4.5mm) was mounted at the outer surface of the dome at an elevation of 45° and allowed to present a light spot to the animals (simulated sun). Tests were carried out with and without the simulated sun switched on (Fig. 1B). Some releases were carried out with the sole dome illuminated (the simulated sun switched off) to exclude any possible use of additional orientation cues, such as the earth's magnetic field. Artificial celestial blue profiles were reproduced by positioning a blue gelatine filter (no 118 Light Blue, λ_{max} = 450 nm, transmittance = 22.16%, SpotLight, Milan, Italy) on the half of the tip of the fibre light guide illuminating the dome. To exclude the possible use of intensity information as orientation cues (radiance gradient), differences in light radiance between the two halves of the dome were reduced by placing two neutral density filters (no 298, transmittance = 69,3% and no 209, transmittance = 51.2%, Illuminoservice, Bologna, Italy) on the other half of the tip of the fiber superimposed. This arrangement produced a spectral gradient across the dome's inner surface but not any absolute intensity differences: thus the only orientating cue could be a spectral gradient across the artificial sky. To compare the artificial spectral gradient with the natural one, disregarding the natural radiance gradient, spectra achieved with outdoor measurement of the open sky were normalized to the light radiance pattern and elaborated according to blue and green sensitivity as explained below. The experiments with the spectral gradient stimulus were carried out in the morning, around noon, and in the afternoon. Thus, if the sandhoppers use spectral information as an orientation cue, they should orientate toward the blue-rich hemisphere in the morning, along the theoretical separation line between the two hemidomes at noon, and away from the blue-rich hemisphere in the afternoon. Prior to each experiment, the position of the blue filter and the azimuth of the simulated sun were adjusted according to the azimuthal position of the "real" sun at the respective time of day. (Fig. 3B-D)

Fig. 1C shows the artificial radiance profile recorded with respect to the natural one measured under clear sky conditions. The curve is relatively flat in the range of about 70°-100° (it varies by about 20%) compared to the natural one: this radiance difference (20%) is too weak to serve as a compass cue for the sandhoppers (see Fig. 2E, F, H, I: a difference of about 40% and 75% is not sufficient to allow compass information with respect to the radiance gradient). The flat radiance profile in the artificial environment is crucial for the evaluation of sandhoppers response to differences in sky

spectral characteristics. To compare the colour (as perceived by sandhoppers) of the two halves of the artificial sky, we must balance the part of the spectrum, of each half, that falls onto the blue sensitivity and the one which falls in the green sensitivity of *T. saltator* under a constant sky radiance.

If we consider a constant-radiance sky, by the point of view of a sandhopper, the blue-rich hemidome is much brighter than the green-rich hemidome both in nature and under our artificial spectral gradient. To consider this, we calculated the green/ blue ratio (G/B) (Fig. 1D). The curves for the G/B ratio measured in nature and under our artificially generated spectral gradient show a similar shape with relatively more green light in the solar hemidome and relatively more blue light in the antisolar hemidome. Also in this case natural measurements were normalized in order to obtain a constant radiance.

Statistical analysis

Data were analyzed following the methods proposed by Batschelet (1981) for circular distributions. For each distribution, the length of the mean resultant vector and the mean angle were calculated. We used the V-test to establish if distributions differed from uniformity ($P < 0.05$ at least) (Aneshansley and Larkin, 1981). The confidential limits for the mean angles (95% confidence, Batschelet 1981) were calculated to assess the orientation towards the seaward (expected) direction. Comparisons among the number of radially-orientated sandhoppers were made by the G test (Zar, 1984)

Results

Artificial radiance gradients

Sandhoppers tested around local noon, under the artificial radiance gradient corresponding to the profile of the clear natural sky orientated significantly toward the seaward direction of their home beach. The mean resultant vector was deflected from the expected direction by only 23° (Fig. 2C).

When the sandhoppers orientation behaviour was tested under weaker radiance gradients, attenuated by 40%; (Fig. 2D, E, F) or 75% (Fig. 2G, H, I), individuals did not exhibit directional choices in accordance with the expected direction; in both cases, they showed a positive phototactic orientation behaviour toward the solar hemidome (Fig. 2F, I).

The number of the radially-orientated sandhoppers out of the number of individuals released is significantly higher in tests performed under the artificial gradient of radiance not attenuated (corresponding to the natural one) than in tests carried out under attenuated gradients (Fig. 4A, G = 13.100, df = 2, P < 0.01, G test).

Artificial spectral gradient

In the control experiment (Fig. 3A) under an illuminated dome that did not provide any visual cue as an orientation reference, (i.e. a simulated sun or intensity radiance or spectral gradient), the sandhoppers were not statistically orientated (the distribution is not statistically dissimilar from uniformity). Thus, only 27% (n = 8/30) of the released individuals were radially - orientated. (G = 6.683, df = 1, P < 0.01, G test)

Sandhoppers tested under the artificial spectral gradient, combined with a simulated sun illuminated were significantly orientated. Irrespective of the time of day tested, the mean directions were in agreement with the seaward direction of their home beach (Fig. 3B-D) deviated by about 2° - 10° from the expected direction. In addition, most of the sandhoppers (80%, n = 210) made a clear directional choice (Fig. 4B white bars): the ratio of orientated animals released at noon and afternoon were about 95% and 94%, respectively. However, only about 50% of the sandhoppers released in the morning are radially orientated.

In experiments carried out under the artificial spectral gradientwithout the simulated sun (Fig. 3E, F, G), the sandhoppers kept mean directions in good agreement with the expected seaward direction at any time of the day: the deviation of the mean directions from the expected direction was 11° in the test performed in the morning, 25° at noon, and 15° in the experiment conducted in the afternoon (Fig. 3E-G). In all cases, the ratio of orientated animals were about 45% out of the total number of individuals released (n = 203, Fig. 4B, black bars).

In the noon and afternoon releases, the frequencies of radially-oriented individuals are significantly higher in the test conducted with the simulated sun illuminated with respect to those carried out in conditions of simulated sun switched off (meridian tests: G = 31.192, df = 1, P < 0.001; postmeridian tests: G = 41.779, df = 1, P < 0.001, G test). This was not the case in the morning. There, the proportion of orientated animals was similar irrespective of whether the simulated sun was added or not to the spectral gradient stimulus (G = 0.450, df = 1, P = NS, G test).

Discussion and Conclusions

Our results confirm the use of the skylight radiance gradient as a compass cue in the zonal orientation of *T. saltator*. Sandhoppers released under the artificial radiance gradient that matched the natural intensity gradient on a cloudless day orientated correctly toward the seaward direction of their home beach (Fig. 2A-C). Instead, the sandhoppers lost their ability to use this celestial cue as an orientation reference when released under artificial radiance profiles that exhibited a weaker intensity gradient than the natural gradient (Fig. 2D-I). The significant reduction of the proportion of radially orientated sandhoppers further confirmed the inability of the sandhoppers to orientate according to attenuated radiance patterns (Fig. 4A). Indeed, as previously stated, the use of skylight

radiance gradient could be used by *T. saltator* to orientate along the sea-land axis of the beach even when the sun is not directly visible (Ugolini et al., 2009, 2012). We can hypothesize that in natural conditions this can occur in the presence of fog or cloud cover of the sky, the spectral gradient being absent or very reduced.

Results obtained in experiments carried out under the artificial spectral gradient (Fig. 3) represent the first evidence on the use of the celestial spectral gradient as a compass cue in *T. saltator*. In fact, sandhoppers performed time-compensated directional choices and kept the correct seaward directions of their home beach independent of the time of release. The uniform distribution obtained releasing sandhoppers with only the dome illuminated, in absence of the simulated sun and radiance and/or spectral gradients (Fig. 3A) demonstrates that *T. saltator* does not use the natural magnetic field as a compass reference under these conditions at least.

It is important to note that the spectral gradient was the only available cue in the experiments where the simulated sun was switched off. Thus, the reduction of the number of radially orientated individuals could be caused by the absence of any further reliable cues, such as the substrate slope and the surrounding landscape (Ercolini and Scapini, 1974; Craig, 1973; Hartwick, 1976, Ugolini et al., 1986, 2006; Ugolini, 2014; Ugolini and Ciofini, 2016). It remains an open question why the sandhoppers exhibited a relatively low number of radial individuals (50%) registered in the morning releases with the artificial sun switched on (Fig. 4B), not statistically different from the frequency registered in the morning releases with the artificial sun switched off (43%). We can hypothesize that it could be due to the conflict between the use of the sun compass and a positive phototactic tendency: in fact, in contrast to the other test conditions, in the morning the azimuth of the sun (= 105° - 110°) and the seaward direction (= 265°) are almost in opposite directions.

Based on ethological and neuroanatomical investigations it is known that the polarization pattern is detected by a specialized region of the compound eye (Labhart and Meyer 1999), while the main retina perceives the spectral and intensity information of the sky in insects (el Jundi et al. 2014b). Information provided by these skylight references are then integrated in the same network in the central nervous system to generate a consistent compass signal (Brines and Gould, 1979; Edrich et al., 1979; Rossel and Wehner, 1984; Wehner, 1989; el Jundi et al., 2014b, 2015a, b, 2019). Previous investigations (Pardi and Papi, 1953, Ugolini, 2013) revealed that *T. saltator* perceives the skylight polarization but it does not use the pattern of skylight polarization as a compass reference. Moreover, it is not yet clear which advantage it has for the sandhoppers to perceive wavelengths in the UV-blue range.

As hypothesized by Coemans et al (1994) it is possible to speculate that the UV sensitivity is part of

a colour processing system well adapted to derive the position of the sun from the use of skylight spectral contrast. According to this hypothesis, we hypothesize that the capability of *T. saltator* to detect the light in the UV range can be ecologically advantageous to render more reliable its overall compass system. However, *T. saltator* perceives UV-blue wavelengths mainly with the lower part of the eye, while the upper part presents photopigments both for the UV-blue and for the green (Ciofini et al., 2020). Therefore, we can imagine that the radiance and spectral gradients are perceived with the upper (dorsal) part of the eye while the UV-blue sensitivity of the lower part is more related to the identification of the sea hemicycle, given the importance of the perception of the coloured landscape (Ugolini et al., 2006) and not only of the contrast between sea and land hemicycle in the zonal recovery of *T. saltator*.

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Competing interests

No competing interests declared

Author contributions

All the authors contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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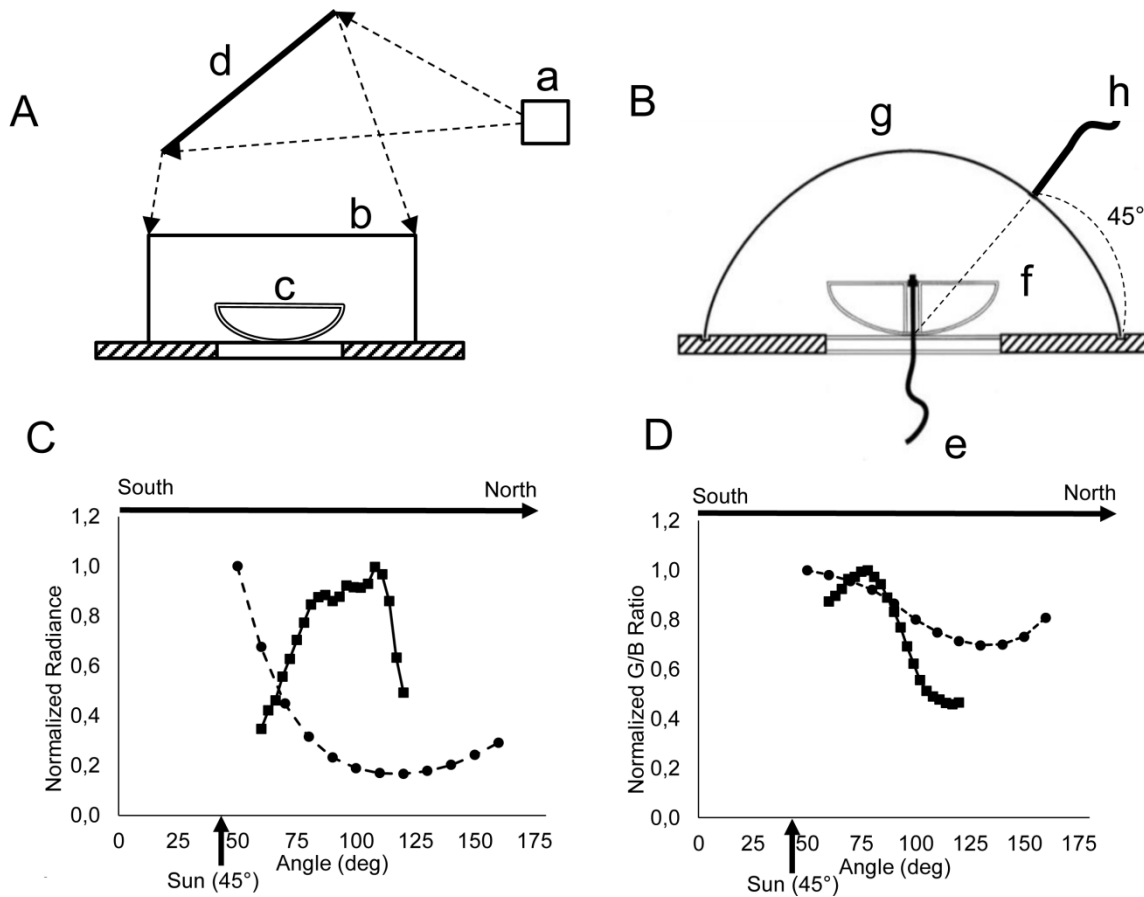**Zar, J. H.** (1984). *Biostatistical Analysis* (2nd ed.). Englewood Cliffs, NJ, Prentice Hall.

Figures

Fig. 1. Devices used in the experiments and spectroradiometric characterization of artificial and natural sky. A, schematic representation of the device used in experiments with the artificial radiance gradients. The image of artificial sky is projected by a video-projector (a) on a circular opaline Plexiglas plate (b) placed over the bowl containing the animals (c). A mirror (d) placed at 45° allows the vertical projection of the artificial sky. B, schematic representation of the device used in the tests with artificial spectral gradient. A fibre-optic illuminator lighting the internal surface of opaline dome by (e) a fibre bundle; (f) bowl containing sandhoppers, (g) Plexiglas opaline dome and (h) fiber bundle simulating the sun (45° of elevation). A video camera was placed under the bowl to record freeze-framed images. C, Radiance recorded under the dome; B, in artificial (solid line) and natural conditions of clear sky (dotted line) as a function of the angle along solar meridian. D, Ratio between green and blue stimulation (G/B) as a function of the angle (from South to North) in the dome (artificial conditions, solid line) and natural conditions (dotted line) along solar meridian. The black arrows on the top in C and D indicate the direction from South to North of the measurements along the solar meridian. The sun elevation is shown on the X axis (see also Fig. 1B). In the solar hemidome there is more green while in the opposite there is more blue. The natural and artificial conditions show similar shape.
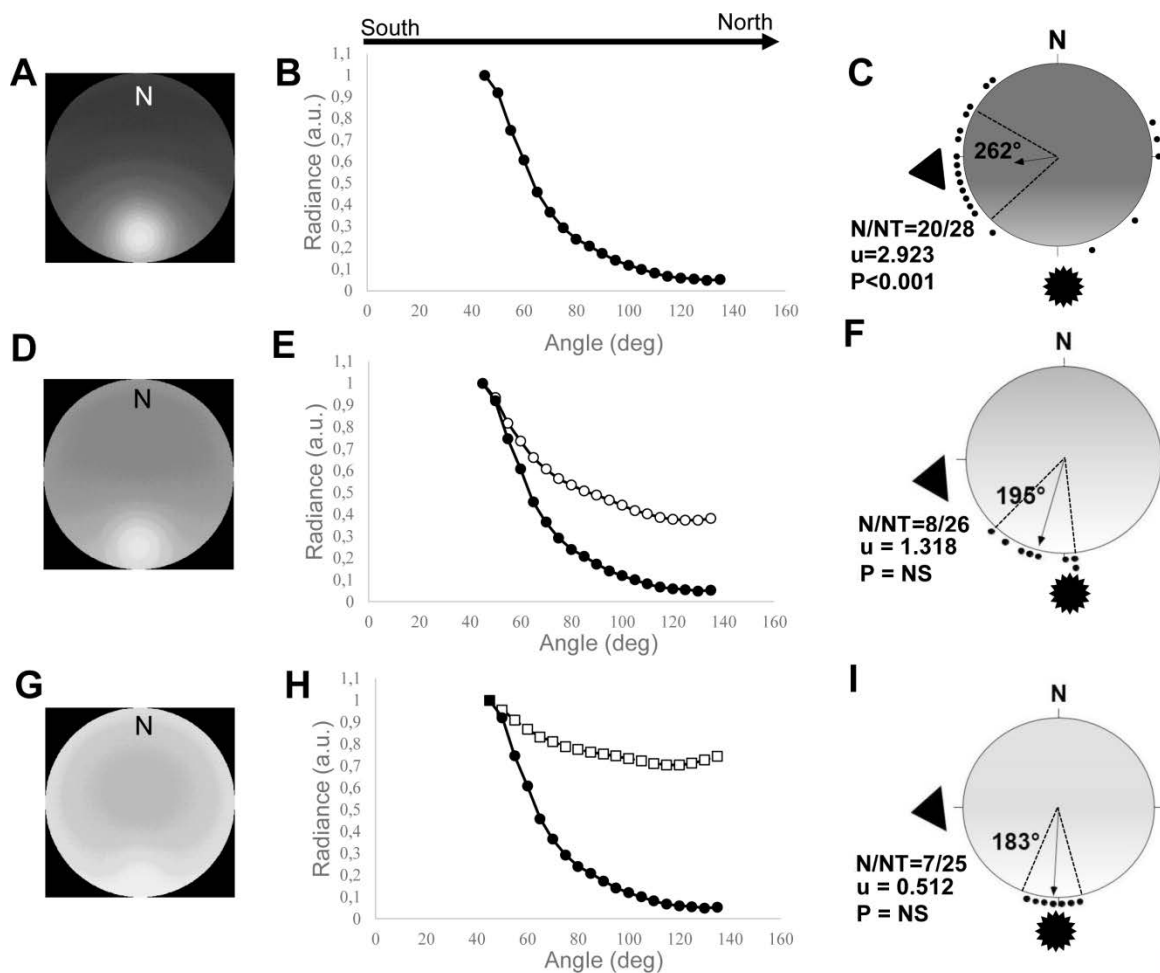
Fig. 2. Artificial radiance gradient experiments. Images of radiance gradients (A, D, G) and relative profile (B, E, H) as a function of the angle along solar meridian (the black arrow on the top in B indicates the direction from South to North of the measurements along the solar meridian). Three artificial gradient images were created: A, reproducing the natural gradient, D and G gradients attenuated by 40% and 75%, respectively. C, F, I, circular distributions corresponding to the different gradients. N, North; black arrows inside the distributions, mean vector and angle (the length of the mean vector ranges from 0 to 1 = radius of the circle); the two dashed lines represent the confidence limits for the mean angle (95%) ; black dots, sandhoppers' directions (each dot corresponds to the direction of one individual); black triangles outside the distributions, seaward direction of their home beach. The symbol of the black sun indicates the solar azimuth at the time of releases even though the sun is not visible. N/NT, number of radially-orientated sandhoppers out of the total individuals tested. The values of the V-test, u, with their probability level, P, are also given.
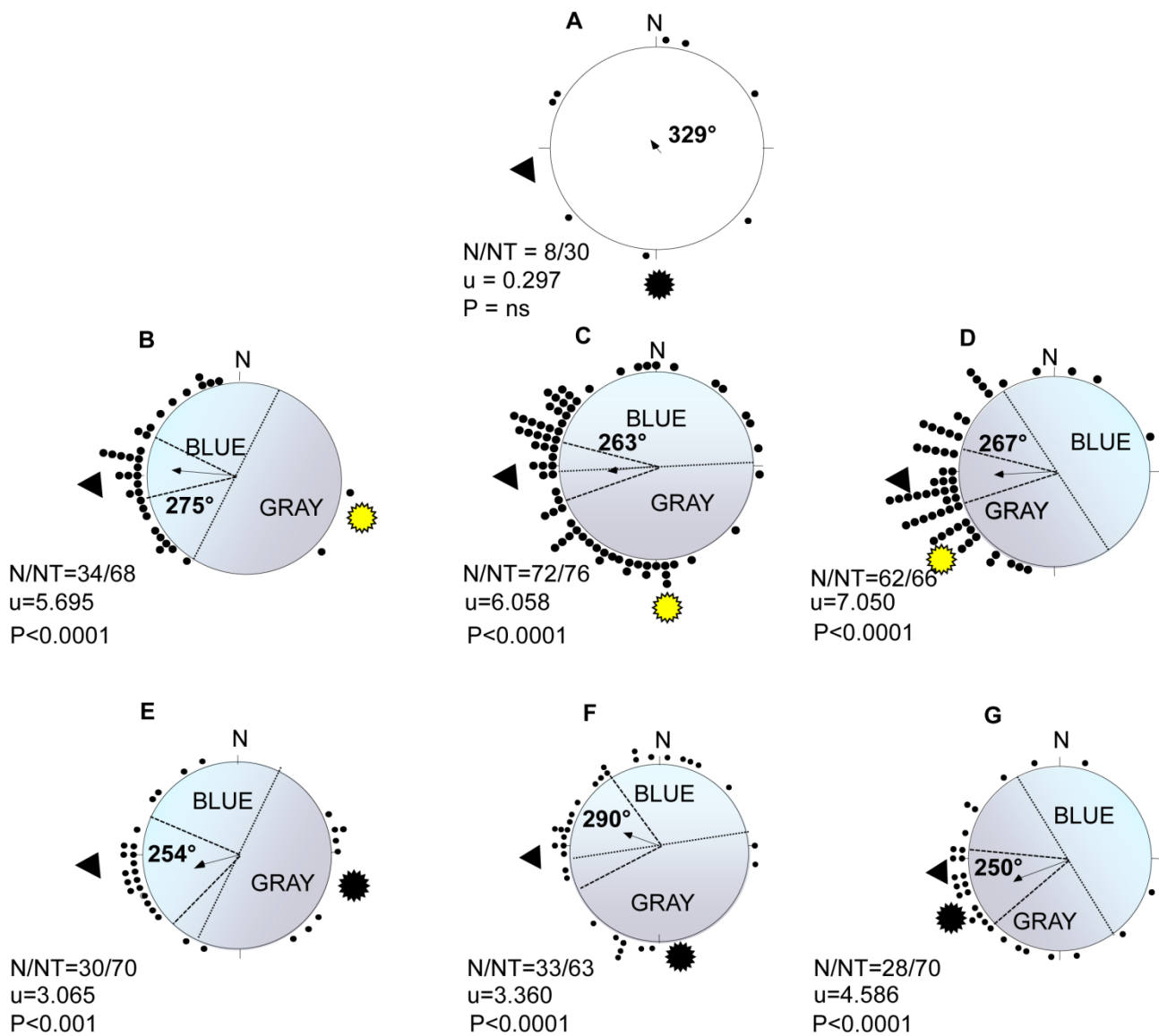
Fig. 3. Artificial spectral gradient experiments. A, control test, the artificial sun swithced off, no kind of gradient. B-G, tests carried out at three different hours of the day with artificial spectral gradient. B-D tests with the artificial sun on. E-G tests with the artificial sun off. The position of the artificial blue-gray spectral gradient varies in accordance with the solar azimuth (symbol of the sun outside the distributions). For further explanations see Fig. 2.
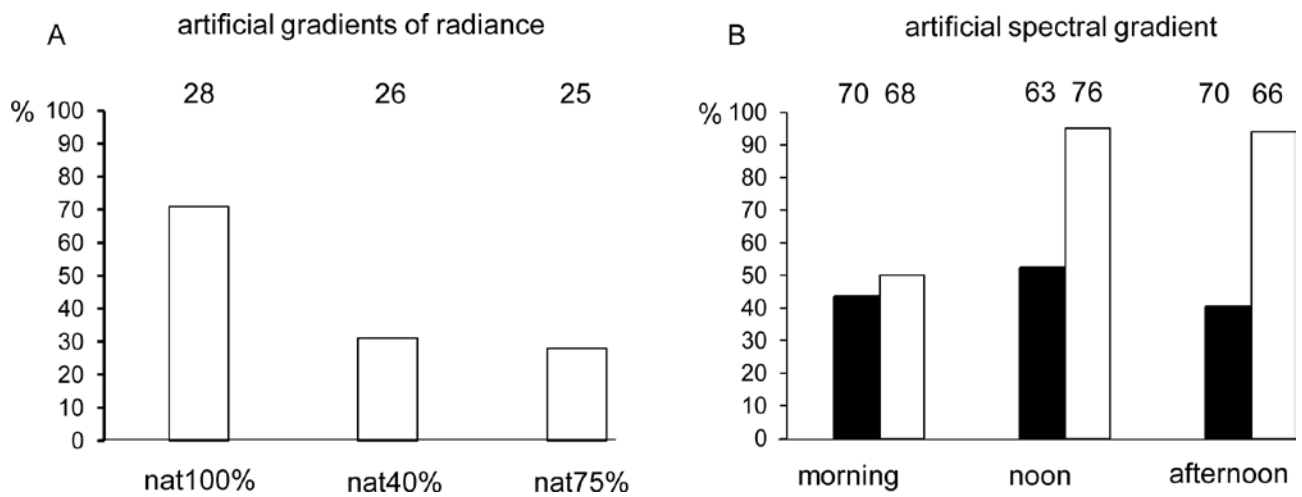
Fig. 4. Frequencies of radially-orientated sandhoppers. A, tests carried out under artificial gradients of radiance corresponding to the natural one (nat 100%), reduced by 40% (nat 40%), and 75% (nat 75%). B, tests with artificial spectral gradient. White bars, frequencies recorded with the artificial sun on at three different hours of the day; black bars, frequencies recorded with the artificial sun off. The numbers above the bars indicate the total number of sandhoppers tested.