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Interactions among celestial orienting factors and their functioning in supralittoral crustaceans

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SUMMARY

ABSTRACT

Behavioural investigations conducted since the Fifties have revealed that the amphipod *Talitrus saltator* can rely on both the sun and the moon as compass cues in its zonal recovery; recently, evidence for discrete endogenous oscillators underlying its time-compensated solar and lunar orientation has been also given. *T. saltator* is the first species shown able to obtain compass information by using only the skylight intensity gradient. Instead, it does not rely on the celestial polarization pattern despite its sensitivity to polarized light. Although discrete receptors detecting UV-blue and green light have been identified within its compound eye, neither the capability of this species to use the spectral pattern of the sky nor the regionalisation of its visual pigments (eventually indicating the existence of a DRA) has been investigated. Furthermore, investigations on the structure of its compound eye conducted so far are quite scarce despite the importance of the vision in the perception of orienting stimuli. Evidence for solar and lunar orientation has been provided also in the isopod *Tylos europaeus*. However, as opposed to its ability to orientate to the sun, its moon compass-based orientation has not been confirmed.

The aims of this work are: 1) to deepen our knowledges on the use of the celestial gradients by *T. saltator*, 2) to evaluate the regionalization of its visual capabilities, 3) to assess the optical and functional structure of its compound eye, 4) to investigate the anatomical localisation of the time-keepers regulating the sun and the moon compass mechanisms, 5) to assess the existence of antennal time-keepers involved in celestial orientation and 6) to confirm the capability of *T. europaeus* to orientate to the moon.

In this work, the first evidence for the use of the celestial spectral gradient as a compass cue by *T. saltator* was obtained. The skylight intensity profile has also been confirmed to constitute a reliable orienting reference and it has been shown that it exists a minimum threshold of the gradient effectively recognised and used. Instead, tests carried out did not point out a clear spatial distribution of the photoreceptors within the eye of this species. However, it has been revealed that the dorsal edge of the eye plays an important role in the perception of celestial factors. These results, along with evidence of straight ommatidia occurring in this area of the eye, suggest a regionalisation of the visual capabilities in *T. saltator* and are in agreement with the existence of a DRA. Furthermore, it was shown that this species mainly possesses hook-shaped ommatidia (except for the dorsal region of its eye) and it was suggested that their photoreception efficiency was enhanced by reflecting pigment cells localized between them. Moreover, it was found that the oscillators underlying the sun and the moon compass mechanisms are localised in separate localities. In fact, the antennae seem to be the anatomical site of the time-keepers responsible for the lunar orientation (although our results suggest that timing inputs from these oscillators are downstream integrated), whereas those involved in solar orientation are located elsewhere (probably in the brain). Intriguingly, present work provided first molecular evidence for time-keepers in *T. saltator* by revealing rhythmicity in the expression of core genes in both brain and antennae (thus supporting the existence of oscillators in these appendages). Finally, it has been fully confirmed the capability of *T. europaeus* to orientate under the moon and provided partial evidence for discrete time-keepers underlying the functioning of the sun and the moon compass systems in this species.

CHAPTER 1

INTRODUCTION

1 Zonal recovery in littoral species

Littoral ecosystems, like other ecotonal environments, are subjected to abrupt changes in physical and chemical parameters. Moreover, periodic (tidal and seasonal) and aperiodic variations contribute to render these environments highly dynamic. To avoid potential harmful effects caused by unfavourable conditions it is crucial for organisms inhabiting such ecosystems that have evolved specific adaptions (behavioural and/or physiological). For instance, many species orientate along the sea-land axis (Y-axis) of the shore, perpendicular to the shoreline (X-axis), in order to remain in the zone characterised by optimal conditions for their survival (see Pardi & Ercolini, 1986 for a review). These strategies of zonal recovery are based on the use of multiple orienting factors belonging to two main categories: local and general cues

Local references directly indicate the direction animals must orientate toward. For instance, the slope of the substratum and/or the vision of the surrounding landscape constitute reliable orienting factors for some species of amphipod such as *Orchestoidea curniculata, Talorchestia longicornis, Talorchestia martensii* and *Talitrus saltator* and the isopod *Tylos punctuatus* (Hamner et al., 1968; Craig, 1973; Ercolini & Scapini, 1974; Hatwick, 1976; Ugolini et al., 1986, 2006; Cohen et al., 2010; Ugolini, 2014; Ugolini & Ciofini, 2016).

Instead, to use general cues is far more complicated since animals have to be able to compensate for their position in order to assume an effective direction and reach their preferred zone. In particular, celestial orienting factors exhibit azimuthal variations across the sky during the 24 h period and thus their use requires adequate chronometric compensatory mechanisms to maintain a constant direction. However, celestial orientation is highly widespread among littoral (as well as riparian) arthropods exhibiting strategies of zonal recovery. For instance, sun compass-based orientation is shared by many species belonging to different taxa such as arachnids (*Arctosa* spp, Papi, 1955a, b 1959; Tongiorgi, 1961; Papi & Tongiorgi, 1963), insects (coleoptera carabids, Papi, 1955c; Costa et al., 1982, tenebrionids, Pardi, 1956, staphylinids, Ercolini & Badino, 1961; Ercolini, 1963; Ercolini & Scapini, 1976a, orthoptera, Felicioni & Ugolini, 1991; Ugolini & Felicioni, 1991 and dermaptera, Ugolini & Chiussi, 1996) and crustaceans (decapoda, Shöne, 1963; Herrnkind, 1972, 1983; Ugolini et al., 1988, 1989; Goddard & Forward, 1989, isopoda, Pardi, 1955, 1963; Ugolini & Pezzani, 1993; Ugolini et al., 1995, 1997 and amphipoda, Pardi & Papi, 1953; Ercolini, 1960, 1964a, b).

Celestial orientation is not always possible at equatorial latitudes because of the high azimuthal speed and the reduced zenithal distance of the astronomical bodies occurring in certain periods of the day and the year (see Braemer, 1960; Ercolini, 1964a, b; Ugolini & Pardi, 1992). Some equatorial supralittoral amphipods have been shown to be also able to orientate according to another general orienting factor, the geomagnetic field, at least in the periods in which celestial references are difficult to use (Ercolini, 1964a, b; Pardi & Ercolini, 1966;

Pardi et al., 1984, 1988; Ugolini & Pardi, 1992; Ugolini et al., 1999a; Ugolini, 2001, 2002; see also Kenneth & Ernst, 2014).

1.1 Ecological challenges of the semi-terrestrial crustaceans *Talitrus saltator* and *Tylos europaeus*

The sandhopper *Talitrus saltator* is an amphipod widespread along the sandy beaches of northern Europe and the Mediterranean Sea. This species, not being completely adapted to terrestrial environment, evolved crepuscular/nocturnal habits to prevent dehydration. During the day animals live buried in temporary refuges into the belt of moist sand of the shore. At dusk, they emerge on the sand and perform inland (up to 100 metres from the damp belt) excursions; the reverse path following the shortest route (the sea-land axis of the beach) begins in the middle of the night (Pardi & Papi, 1953; Geppetti & Tongiorgi, 1967a, b; Scapini et al., 1992). In populations inhabiting highly tidal shores even seaward excursions have been observed (Williamson, 1951; Edward & Naylor, 1987).

However, both biotic (predators) and abiotic (strong wind or/and tides) disturbing factors can cause active or passive displacements of individuals from the damp belt even during the day and to return as quick as possible to their preferred zone is crucial to escape from death. For this reason, when exposed to conditions of low humidity and high temperature animals promptly orientate seaward whereas submersion induces immediate landward orientation.

In their zonal orientation, sandhoppers can use several cues, both local and general. In particular, *T. saltator* is one of the best-known models among arthropods for studies of celestial orientation. Indeed, since the Fifties there is evidence for its capability to orientate according to the sun (as previously hinted) and the moon (see Pardi & Ercolini, 1986 for a review) and it is one of the few species in which the existence of a chronometrically-compensated lunar orientation has been fully confirmed (Ugolini et al., 1999b, c, 2007a, 2012). Moreover, *T. saltator* is also the sole arthropod known able to obtain compass information by relying exclusively on the skylight intensity gradient (Ugolini et al., 2009).

Even the isopod *Tylos europaeus*, meeting the same ecological challenges of *T. saltator*, performs nocturnal habits and can be forced to leave the moist band of sand (where it normally lives buring daytime) during the day by environmental stressors. The orienting mechanisms underlying the zonal recovery have been scarcely investigated in this species. Nevertheless, it has been demonstrated to possess innate capability of time-compensated solar orientation (Pardi, 1955; Ugolini et al., 1995, 1997) and preliminary evidence for its moon compass-based orientation has been also given (Pardi, 1954a).

2 Celestial orienting factors among arthropods

Besides species inhabiting littoral environments, celestial factors constitute reliable cues for many arthropods with different ecological necessities. As previously stated, their use involves chronometric systems to compensate for their apparent movements across the sky and continue orientating effectively throughout the 24 h period.

First evidence for time-compensated solar orientation in arthropods was given by von Frisch (1950). In his studies on the honey bee *Apis mellifera*, he showed that individuals during their feeding excursions could rely on the sun varying the angle assumed to the astronomical body during the day. In the following years, the existence of mechanisms of sun compass was demonstrated in many other species exhibiting pluridirectional orientation, such as the desert ant *Cataglyphis bicolor* (Wehner, 1972) and the Monarch butterfly *Danaus plexippus* (Froy et al., 2003).

The moon is another celestial factor that could help nocturnal species orientate at night. However, its use as a compass cue determines additional difficulties that animals have to overcome compared to the sun. In fact, the moon is not always visible during the lunar month (new moon phase), it does not show the same shape and each night it rises with a delay of 50 minutes with respect the previous one. Moreover, its hourly azimuthal variations are wider that those exhibited by the sun. To date, time-compensated lunar orientation has been fully demonstrated only in few species (see Wallraff, 1981; Ugolini et al., 1999b, c, 2007a, 2012; Ciofini & Ugolini, 2017).

Besides the sun and the moon, even other celestial factors have been shown to provide directional (compass) information to several species of arthropods. Indeed, the passage of the sunlight through the atmosphere defines a pattern of polarized light and characteristic profiles of luminance and spectral distribution across the sky concordant with the position of the sun (see Waterman, 1981 for a review). These factors can be detected also in the absence of the direct vision of the sun conferring animals able to rely on them undeniable ecological advantages.

The deviation of sunlight operated by atmospheric particles determines its vibration in only one direction, perpendicular to that of propagation. This phenomenon defines a profile of E-vectors, concentric with the sun, each corresponding to a scattered (polarized) ray (Menzel, 1975; Waterman, 1981; Brines & Gould, 1982; Wehner, 1989; Tovee, 1995; Eguchi, 1999). According to the Rayleigh model (Strutt, 1871), the highest degree of polarization across the sky occurs at 90° with respect the sun and gradually decreases in both directions.

The celestial polarization pattern has been shown to be used as a compass cue by many species. The first investigations on arthropods were conducted by Von Frisch (1949) who showed the ability of *A. mellifera* to perceive and rely on this factor. Later, further studies revealed a substantial diffusion in its use among taxa (see Hovárth & Varju, 2004 for a review). The directional information provided by this cue seem to be particularly important in the orientation of *Cataglyphis bicolor* since its homing behaviour is affected when the perception of the polarization pattern of the sky is prevented even in conditions of sun visible (Duelli & Wehner, 1973; Wehner, 1992).

Animals perceive celestial polarization mainly in the UV (see Duelli &

Wehner, 1973; Menzel & Snyder, 1974; Waterman, 1981; Stalleicken et al., 2005) although the degree of polarization and the radiance of light in clear sky conditions are lower in UV wavelengths than in longer ones (UV sky-pol paradox). In fact, UV light is less influenced by atmospheric adverse conditions than blue or green light and does not undergo absorption by the foliage (Barta & Hovárth, 2004).

The vision of polarized light is generally mediated by ommatidia occurring in the dorsal edge of the compound eye exhibiting physiological adaptions that optimize their sensitivity to polarization (Dorsal Rim Area, DRA). However, differences in both the anatomical structure and the degree of specialization of the DRA have been pointed out among taxa (see Labhart & Meyer, 1999 for a review). Generally, rhabdoms are shorter and larger than those occurring in other regions of the eye since rhabdomeres are shortened to reduce self-screening and enlarged to enhance sensitivity. Moreover, they have a characteristic shape due to the increase of the number of retinula cells and large photoreceptors. To properly detect polarization from different directions, microvillar expansions constituting the same rhabdom are distributed in two sets orientated orthogonally each other. In fact, the sensitivity to polarized light is highest when the long axis of the photoreceptors is parallel to its plane of oscillation. Sensitivity is further enhanced by the alignment of the microvillar expansions along the rhabdom. Furthermore, the shape of the visual field of the DRA is elongated since ommatidia present in this region exhibit optical axes orientated upward. Polarization-sensitive photoreceptors are typically homochromatic to avoid interferences with spectral information.

In species like the bee *Apis mellifera* (Wehner & Strasser, 1985), the cricket *Gryllus campestris* (Labhart et al., 1984) and the ant *Cataglyphis bicolor* (Duelli & Wehner, 1973; Wehner, 1992), a peculiar spatial distribution of their visual pigments has been revealed. Indeed, photoreceptor perceiving UV light (of course also accountable for the detection of polarization) are mainly located in the DRA whereas those sensitive to longer wavelengths are concentrated in the ventral region of the eye.

The skylight luminance profile is due to the light intensity variations along the solar meridian. Its use as an orienting factor was first supposed by Verheijen (1978) but scarcely investigated (Wehner, 1997; Ugolini et al., 2009; el Jundi et al., 2014). To date, *T. saltator* is the sole species known able to perform effective directional choices relying only on this cue (Ugolini et al., 2009). In fact, the dung beetle *Scarabaeus lamarki* (not exhibiting time-compensated orientation) has been demonstrated to use this reference but only in combination with the polarization pattern (el Jundi et al., 2014). Instead, *Cataglyphis bicolor* when allowed to orientate relying on the luminance pattern does not assume a correct homeward direction and exhibits phototactic responses toward the brighter region of the sky (Wehner, 1997).

The spectral pattern of the sky is characterized by the predominance of shorter (293-460 nm) wavelengths in the celestial hemicycle including the sun ("solar" hemidome) and longer (460-700 nm) wavelengths in the opposite one ("antisolar" hemidome) defining a gradient along the solar meridian (Waterman, 1981; Coeman et al., 1994). Some arthropods, such as *Apis mellifera* (Brines & Gould, 1979; Edrich et al., 1979; Rossel & Wehner, 1984), *Cataglyphis bicolor* (Wehner, 1997) and *Scarabaeus lamarcki* (el Jundi et al., 2015), can orientate by relying on celestial spectral stimuli that are used to discriminate the "sun" and the "antisun" directions.

2.1 Celestial orientation in *T. saltator*

In the Fifties, Pardi and Papi (1952, 1953) provided first evidence for time-compensated solar orientation in this species by performing experiments of sun azimuth deflection, similar to those conducted on the ant *Messor barbarus* by Santschi (1911). They carried out their tests in a confined environment suitable to prevent the perception of other local orienting factors and allowing animals the vision of the natural sky. Individuals exhibited directional choices in accordance with the Teoretical Escape Direction (TED, corresponding to the sea-land axis of the shore) of their home beach in conditions of natural sun visible. Instead, when the sun was screened and reflected by a mirror, animals deflected their directions by assuming an angle to the reflected sun corresponding to that expected to the natural celestial body to orientate effectively. Further experiments conducted by releasing individuals previously subjected to phase-shifted L:D cycles under the sun revealed that in any case sandhoppers exhibited directional choices in agreement with their subjective time (Papi, 1955d; Pardi & Grassi, 1955; see also Ugolini, 2003).

Differently from other species of littoral arthropods, in *T. saltator* chronometrically-compensated solar orientation is innate. Indeed, individuals born in captivity and prevented from the vision of the natural sky and sun until their release are able to orientate toward the same escape direction of expert adults. Solar orientation improves in adults, probably because of the possibility to associate local elements of the beach with compass information provided by the astronomical body (Pardi et al., 1958; Pardi, 1960; Pardi & Scapini, 1983; Scapini et al., 1985). However, sandhoppers (especially young individuals) can modify their innate TED by learning a new escape direction when dislocated in different beaches; this feature allows animals to colonize new shores and thus confers ecological advantages to this species (Ugolini & Macchi, 1988; Ugolini et al., 1991).

Experiments conducted under artificial conditions of illumination revealed that the recognition of the sun is dependent on the intensity of the sources of light simulating the celestial body and sky. In fact, animals perform proper time-compensated solar orientation only if the intensity of the artificial sun and sky exceed 1.1-1.5 $\mu W/cm^2$ and 3-10 $\mu W/cm^2$ respectively (Ugolini et al., 1998; 2005).

Moreover, solar orientation is disrupted when the perception of wavelengths shorter than 450 nm is prevented; in this case, animals exhibit phototactic responses toward the sun (Ugolini et al., 1996).

Even the capability to use the moon as a compass orienting factor was demonstrated through experiments of lunar azimuth deflection (Papi & Pardi, 1953, 1954, 1959; Papi, 1960; Enright, 1972). Also in this case, sandhoppers modified their directional choices and assumed an angle to the deflected moon corresponding to that expected to the natural celestial body to orientate effectively toward their escape direction. Moreover, recent experiments showed that time-compensated lunar orientation in this species is independent of both the natural geomagnetic field and the shape of the moon (Ugolini et al., 1999c).

Lunar orientation in *T. saltator* is innate; in fact, inexpert individuals (born in captivity) when released under the moon are able to carry out directional choices comparable to those exhibited by expert adults belonging to the same population (Ugolini et al., 1999c, 2003).

The recognition of this celestial body is not strictly dependent on its intensity and spectral composition as shown in studies conducted by using artificial sources of illumination to replace the natural celestial body (Ugolini et al., 2005, 2007a).

Recently (Ugolini et al., 2009), it has been demonstrated that the compass information provided by the skylight intensity gradient allow *T. saltator* to orientate correctly. Sandhoppers released in a confined environment under an opaline Plexiglas dome directly illuminated by the sun (reproducing with good approximation the natural intensity pattern of the sky) orientated in agreement with their escape direction. Instead, when tested under the dome with the sun screened (eliminating the luminance gradient along the solar meridian) animals were unable to perform directional choices (no radially-orientated). These results were confirmed in similar experiments conducted at equatorial latitudes where no directional information can be obtained from the luminance profile occurring along the solar meridian when the sun reaches its zenith. In fact, whereas individuals were well orientated when the zenithal distance of the sun was 39°, they were not able to carry out any directional choice when the sun was close to its zenith (zenithal distance = 10°). Further investigations (Ugolini et al., 2012) revealed that the gradient of luminance is also involved in the discrimination between the sun and the moon. Nocturnal experiments conducted in conditions of artificial illuminations showed that animals exhibited correct time-compensated lunar orientation when tested in the absence of the intensity pattern (although the intensity of the source of light simulating the astronomical body exceeded the threshold necessary for inducing sandhoppers to perform solar orientation). Instead, in tests carried out by reproducing an artificial skylight luminance profile, individuals released under the same illumination conditions exhibited directional choices in agreement with the model of nocturnal solar orientation proposed for this species (*Talitrus* model, see Pardi, 1954b and paragraph 2.2.1 of this chapter).

The the celestial polarization pattern does not play any role in the zonal recovery of *T. saltator* although evidence for its capability to perceive polarized light has been provided (Pardi & Papi, 1953; Ugolini et al., 2013). In tests conducted in the Fifties by releasing sandhoppers under a polarizing filter (Pardi & Papi, 1953) a large part of individuals tested was unable to perform directional choices and changed continuously their position. Moreover, animals exhibited clear signals of stress since they tried to hide themselves under the others forming clusters in order to prevent dehydration. However, it was observed that the disposition of the clusters changed according to the positioning of the polarizer and individuals tended to concentrate themselves in the quadrants including the expected direction on the basis of the orientation of the filter. Lately, Ugolini et al. (2013) in similar tests revealed that sandhoppers lost their capability to perform effective directional choices only when the vision of the sun was prevented. Even in this case researchers observed tendencies to form clusters whose disposition varied according to the polarizer positioning.

2.1.1 Visual system and spectral sensitivity of *T. saltator*

The compound, sessile gammarid-like eye of *T. saltator* (diameter ~ 0.6 mm) (Hallberg et al., 1980) is formed by about 300 ommatidia (diameter ~ 35 μm) (Gallien & David, 1936).

Each ommatidium, representing the structural unit of the eye, includes a dioptric and a photoreceptive region.

The dioptric region consists of a hexagonal cornea (15 μm thick) that is part of the cuticle encasing the whole body and a crystalline cone (length ~ 60 μm; maximum diameter = 35 μm, see Ugolini et al., 2010) formed by two cells and attached to the end of the rhabdom.

The photoreceptive region is constituted by a rhabdom formed by the microvillar expansions (rhabdomeres) of five retinular cells (R1-R5). Four of these cells (R1-R4) are large and arranged in two sets (R1-R3 and R2-R4) with their rhabdomeres orientated perpendicularly each other. The fifth cell (R5) is shorter and its rhabdomere is smaller than the other ones contributing the rhabdom.

T. saltator possesses a fused-type rhabdom since rhabdomeres are strictly joined to each other (Ercolini, 1965). Rhabdoms are on average 70 μm long in ommatidia occurring in the central region of the eye whereas they are shorter in those present in the dorsal edge. Moreover, dense pigment granules (diameter = 0.4 μm) surrounding the rhabdom have been observed (Ugolini et al., 2010).

It was supposed (Ercolini, 1965) that the light absorption efficiency of each ommatidium was optimized by straight rhabdoms since such structures could facilitate the passage of the light through the optical system. However, no investigation has been carried out to confirm this hypothesis.

Ommatidia are optically separated from one another through shielding pigments to prevent light from spreading among close units in conditions of intense illumination (apposition eyes) (Ercolini, 1965, see Glanz, 2014 for a detailed description of this visual system). Apposition eye is mainly widespread among diurnal arthropods even if, besides *T. saltator*, even other species exhibiting this anatomical feature are active at night. Moreover, such a specific adaption to high light intensity conditions facilitates the orientation of sandhoppers during the day.

Preliminary investigations conducted to evaluate the spectral sensitivity of this species provided evidence on its capability to perceive short wavelengths (Ercolini & Scapini, 1976b). In fact, in experiments of binary choice assessing their responses to monochromatic stimuli, animals showed a significant phototaxis when irradiated with blue or a green light, whereas orange and red sources of light did not induce any phototactic behaviour. Moreover, in similar tests conducted to evaluate their responses to polychromatic stimuli, individuals showed a clear preference for the sources of light characterized by shorter wavelengths. The sensitivity of *T. saltator* to blue light (λ = 450 nm) was even shown later through electrophysiological recordings (Mezzetti & Scapini, 1995; Ugolini et al., 1996).

The perception of UV light by this species has been long speculated (Ugolini et al., 1993, 1996, 2004). In fact, preliminary electrophysiological investigations suggested a peak of sensitivity at 380 nm (Laffort, 1992). Moreover, as previously stated, experiments of spectral filtering revealed that the chronometrically-compensated solar orientation exhibited by this species was disrupted when wavelengths shorter than 450 nm were blocked (Ugolini et al., 1996) Only recently (Ugolini et al., 2010), has this argument been clarified and the sensitivity of this species to UV light has been fully confirmed. Indeed, tests of binary choice showed significant phototactic responses of sandhoppers when irradiated with UV light,

Electrophysiological recordings revealed also the existence of two discrete photoreceptors accountable for the perception of UV-blue (390-450 nm) and green (500-550 nm) light (Ugolini et al., 2010) supporting previous evidence (Ercolini & Scapini, 1976a). Since the vision of the landscape constitutes a local orienting factor involved in the zonal orientation of sandhoppers (Ugolini et al., 1986, 2006), to perceive both blue and green wavelengths facilitates the discrimination between the seaward (blue) and the landward (green) directions. Moreover, the spectral sensitivity of *T. saltator* potentially allows this species to detect the skylight spectral pattern and thus rely on this factor; however, any investigation has been conducted to verify this hypothesis.

Tests of binary choice (Ugolini et al., 2013) revealed also that sandhoppers are sensitive to polarized light in the blue wavelength range. Indeed, individuals irradiated with blue (λ = 435 nm) light significantly preferred a linearly polarized source of light than a non-polarized one whereas no preference was observed in experiments conducted with white and green sources of light. Since this species can not rely on the skylight polarization pattern in its zonal recovery (Pardi & Papi, 1953; Ugolini et al., 2013), it has been supposed that the capability to detect polarized light could be involved in accentuating the light intensity and/or spectral contrast between the two hemicycles of the sky and thus help sandhoppers use other celestial cues (Ugolini et al., 2013).

Preliminary morphological observations suggested some anatomical differences in the structure of ommatidia located on the dorsal edge of the eye with respect those occurring in the rest of the eye (Hariyama, personal communication). These results are concordant with the existence in *T. saltator* of a Dorsal Rim Area (responsible for the detection of polarized light) comparable to that occurring in other arthropods. However, further investigations aimed to assess the eventual regionalisation of the visual capabilities within the eye of this species could give support to this supposition.

2.2 Endogenous oscillators

The chronometric mechanisms responsible for celestial orientation are finely regulated by endogenous oscillators that measure the passage of time ("continuously-consulted" clocks) allowing individuals to correctly compensate for the azimuthal variations of the orienting cues and assume constantly effective directions (see Palmer, 1976; Saunders, 1977 for a review).

Endogenous time-keepers drive a wide multiplicity of biological processes (physiological, metabolic and behavioural) occurring rhythmically over time independently of the degree of complexity of organisms.

Conversely to biological rhythms strictly set by exogenous timing factors, those controlled by internal clockworks persist under constant conditions (i.e. in the absence of time-cues) with a typical periodicity genetically determined (free-running period, τ) (see Saunders, 1977 for a review). First evidence for the existence of self-sustained rhythms was provided by de Mairan (1729). Exposing specimens of *Mimosa pudica* to constant darkness (DD), he demonstrated that the opening-closing cycle of the leaves continued even in the absence of any stimulus provided by the external photoperiod.

Biological rhythms are classified according to their periodicity (ranging from milliseconds to years) into:

- Ultradian rhythms: period < 24 h
- Circadian rhythms: period ~ 24 h
- Infradian rhythms: period > 24 h

Biorhythms generally allow organisms to adapt their activities and physiological processes to external geophysical cycles anticipating predictable changes that occur in their environment conferring them ecological advantages. As this regard, the main rhythms related to external oscillations are:

- Circatidal rhythms (period ~ 12.4 or 24.8 h): typical of organisms inhabiting intertidal areas subjected to strong fluctuations of physical or chemical sources of stress (such as temperature, salinity, humidity and mechanical agitation) due to the tidal cycle. Therefore, the development of biorhythms with a periodicity similar to that exhibited by tides helps animals minimize potential harmful effects of environmental stressors (see Naylor, 2010 for a review).
- Circadian rhythms (period ~ 24 h): very widespread among organisms, allow the synchronization to the light:dark (L:D) and temperature

cycles. These rhythms are the more deeply investigated and therefore the best-known (e.g. see Pittendrigh, 1960; Ashoff, 1965, 1981; Saunders, 1977; see also Vitaterna et al., 2001 for a review).

Rhythms exhibiting circadian periodicity have been revealed even in animals inhabiting arctic latitudes such as rodents (Swade & Pittendrigh, 1967) and reindeer (van Oort et al., 2005). However, these species perform rhythmic activities only during the periods of the years in which substantial photoperiodic variations occur.

Of course, even the compensatory mechanisms responsible for time-compensated solar orientation have circadian nature.

- Circalunar rhythms (period ~ 29 days): mainly widespread among littoral species needing to adapt their activities to the spring-neap tidal cycles (see Naylor, 2010 for a review).

Moreover, although no definitive evidence has been provided, circalunar rhythms could potentially allow organisms exhibiting time-compensated lunar orientation to overcome the difficulties met in the correct use of the moon as a compass cue (i.e. its variations in shape, visibility and azimuth at correspondent times during the lunar month).

- Circannual rhythms (period ~ 1 year): mainly found in species inhabiting temperate latitudes (subjected to seasonal fluctuations of temperature and L:D cycle) that exhibit behavioural activities and/or physiological processes concentrated at specific times of the year (see Saunders, 1977 for a review).

Free-running periods exhibited under constant conditions (τ) are usually slightly different with respect the periodicity of the external geophysical cycles (T, Zeitgeber Time) and are subjected to a high inter- and intra-specific variability. Environmental factors (Zeitgebers) are responsible for their synchronization. The L:D cycle is the more common Zeitgeber entraining circadian rhythms to the 24 h period (Hall, 2003), but also temperature, social interactions and food availability fulfil the same function in same species (see Saunders, 1977). The experimental exposure to L:D or temperature cycles phase-shifted with respect the natural ones induces corresponding phase-shifting of the rhythms as well as (artificial) cycles shorter or longer than those occurring in natural conditions determine their shortening and lengthening, respectively (see Aschoff, 1981 for a review).

Moreover, biological rhythms are characterized by their independence of the external temperature (i.e. free-running periods are not substantially affected by temperature fluctuations). This feature constitutes an important adaptative advantage for heterothermic species that can not regulate their body temperature.

Endogenous time-keepers are part of complex systems defined biological clocks. In general, the term biological clock refers to an ensemble consisting of three major components:

- Input pathway transmitting exogenous timing information provided by environmental stimuli (Zeitgebers) that entrain the endogenous oscillator

- Self-sustaining oscillator localized in specific cells (pace-maker)
- Output pathway synchronized by the oscillator and driving biological processes that occur rhythmically.

2.2.1 Molecular functioning of endogenous oscillators

Several studies conducted to assess the biochemical mechanisms regulating self-sustaining circadian time-keepers identified transcriptional-translational positive and negative feedback loops directly responsible for their proper functioning. Comparative studies revealed that core clock elements are frequently conserved across taxa (see Young & Kay, 2001 and Kyriacou et al., 2008 for a review).

In *Neurospora crassa*, the heterodimer formed by the proteins WHITECOLLAR1 (WC1, a light-activated photoreceptor) and WHITECOLLAR2 (WC2) acts as a transcription factor of the gene *frequency* (*frq*). The peptide FRQ migrates into the nucleus inhibiting the complex WC1-WC2 and thus its own transcription; the degradation phosphorylation-dependant of FRQ allows the cycle to start again (Aronson et al., 1994; Froelich et al., 2003). In this species, it has been even identified a second oscillatory mechanism, autonomous from the transcriptional-translational regulatory loop described above, defined FLO (*fqr*-less oscillator) (see Iwasaki & Dunlap, 2000) and entrained by temperature cycles (Pregueiro et al., 2005).

Studies focused on the functioning of the central time-keeper of *Drosophila melanogaster* (see Allada & Chung, 2010 and Mazzotta & Costa, 2016 for a review) revealed the heterodimer CLOCK (CLK)-CYCLE (CYC) induces the transcription of the genes *per* (*period*) and *tim* (*timeless*) (by binding the e-box situated in their promoter), whose products dimerize and migrate into the nucleus where they suppress their own transcription by interacting with the complex CLK-CYC. The degradation of PER is accelerated by the addition of phosphate groups operated by DOUBLETIME (DBT); however, as a part of the complex PER-TIM, PER is resistant to this process. The activation of the photoreceptor CRY2 (CRYPTOCHROME2) induced by blue light (through a conformational change) let this protein to interact with TIM, causing its degradation. As a consequence, PER is susceptible to the phosphorylation operated by DBT (Fig. 1A).

In other species of insects, different regulatory mechanisms have been revealed (see Sandrelli et al., 2008 and Tomioka et al., 2012 for a review). For instance, in the honey bee *A. mellifera* (Rubin et al., 2006), the heterodimer CLK-CYC drives the transcription of the genes *per, tim* and *cry2* (the latter a vertebrate-like cryptochrome). The products of these genes form complexes that migrate into the nucleus where CRY2 inhibits CLK-CYC. Instead, in the Monarch butterfly *D. plexippus*, besides a vertebrate-like cryptochrome (CRY2) involved in the inhibition of the CLK-CYC complex, also a *Drosophila*-like cryptochrome (CRY1), implied in the synchronization of the oscillator to the external photoperiod, has been identified (Zhu et al., 2005, 2008; Markert et al., 2016)

(Fig. 1B).

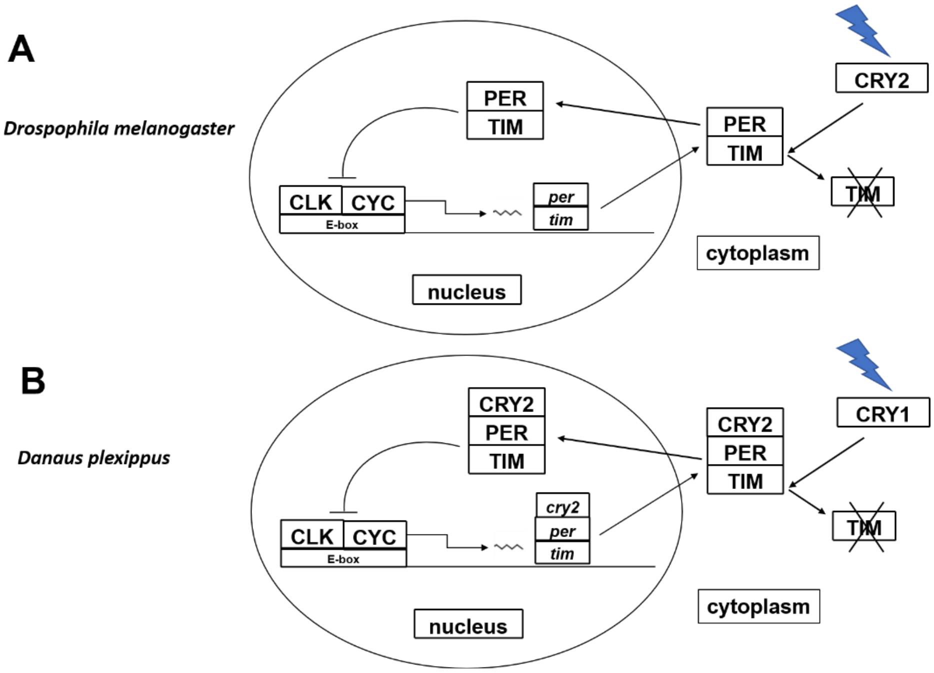

Fig. 1. Schematic representation of the molecular functioning of endogenous oscillators in *D. melanogaster* (A) and *D. plexippus* (B).

Transcriptional-translational feedback loops underlie also circadian oscillators occurring in mammalians (see Ko & Takahashy, 2006 for a review). Basically, the heterodimer CLK-BMAL1 induces the transcription of the genes *per* (*per1*, *per2*, *per3*) and *cry* (*cry1*, *cry2*) whose cognate proteins form complexes (PER-CRY) that migrate into the nucleus and interact with CLK-BMAL1 inhibiting their own transcription. The dimer CLK-BMAL1 is also responsible for the transcription of two retinoic acid-related orphan nuclear receptors, *rev-erbα e rorα*. REV-ERBα and RORα, respectively a repressor and an activator of the gene *bmal1*, finely regulate the transcription of the gene competing to bind specific element present in its promoter.

Besides autoregulatory feedback loops, even post-transcriptional and post-translational events are involved in the regulation of endogenous oscillators at a molecular level (Lakin-Thomas, 2006; Merrow et al., 2006; Mehra et al., 2009). For instance, as described above, the phosphorylation of FRQ and PER occurring in *N. crassa* and *D. melanogaster* respectively promotes their degradation. In mammalians, it has been shown that the proteins Casein Kinase 1δ and Casein Kinase 1ε (CK1δ and CK1ε, the latter homologue of *Drosophila* DBT) play a fundamental role in the turnover of core clock elements (Lowrey et al., 2000; Akashi et al., 2002; Eide et al., 2002; Xu et al., 2005). Their importance in the correct functioning of clock mechanisms has been demonstrated in some studies revealing that mutations of the genes encoding these proteins determine shorter circadian periods (Lowrey et al., 2000; Xu et al., 2005).

In general, post-transcriptional and post-translational events are fundamental for the maintenance of pace-makers' rhythmicity since they ensure the proper time-delay between discrete steps of the cycle avoiding the achievement of a steady state (Gallego & Virshup, 2007; Gatfield & Schider, 2007; see also Kyriacou et al., 2008).

Recent investigations revealed that self-sustaining time-keepers are not necessarily based on transcriptional-translational feedback loops. In hepatic cells of mice (Reddy et al., 2006) and human red blood cells (O'Neill et al., 2011) peroxiredoxins, suggested also as key markers of organisms exhibiting circadian rhythms (Edgar et al., 2012), undergo circadian cycles of overoxidation depending only on post-translational regulatory systems. A similar mechanism has been identified also in the unicellular pico-eukaryotic alga *Ostreococcus tauri* (O'Neill et al., 2011). Moreover, it has been demonstrated that incubating the gene products of the operon KaiA/KaiBC (underlying the functioning of cyanobacterial pace-makers) with ATP the cycle of phosphorylation of KaiC continues to exhibit robust circadian oscillations (Nakajima et al., 2005).

2.2.2 Anatomical organization of endogenous oscillators

In pluricellular organisms, time-keepers regulating distinct behavioural or physiological processes occur in discrete anatomical districts, conferring peripherical tissues specific clock functions. Indeed, several studies conducted on arthropods revealed, besides "master" oscillators located in the central nervous system and governing mainly their behavioural rhythms (see Zhang & Emery, 2012 for a review), the existence of multiple peripherical time-keepers (see Tomioka et al., 2012 for a review). For instance, in *D. melanogaster* self-sustaining oscillators have been identified in several districts, like proboscis, Malpighian tubules, legs, wings and antennae (Hege et al., 1997; Plautz et al., 1997). These time-keepers are independent of the central clock since canonical clock genes continue to be expressed rhythmically even in isolated tissues (Hege et al., 1997; Plautz et al., 1997) and they can be entrained to light and temperature cycles *in vitro* (Levine et al., 2002; Glaser & Stanewski, 2005). Furthermore, it has been shown that oscillators located in Malpighian tubules, as well as the central clock, are synchronized to the external L:D cycle by the photoreceptor CRY2 (Ivanchenko et al., 2001).

In some insects, such as the cricket *Gryllus bimaculatus* (Uryu & Tomioka, 2010) and the cockroack *Leucophaea maderae* (Page & Koelling, 2003; Saifullah & Page, 2009), it has been revealed that peripherical time-keepers, even in the case they have the competence for autonomously self-sustaining, are in fact driven by the central oscillator. Further investigations demonstrated that in these species clock-entrainment is mediated solely by retinal photoreceptors (Tomioka & Chiba, 1984; Nishiitsutsuji-Uwo & Pittendrigh, 1968; see also Sandrelli et al., 2008 and Tomioka et al., 2012 for a review).

Studies aimed to assess the relationships between discrete oscillators have been conducted even on some species of crustaceans. In particular, in crayfish (*Procambarus clarkii*, *Procambarus bouvieri* and *Cherax destructor*) no master clock has yet been identified but evidence for autonomous separate time-keepers interacting each other has been obtained (Arechiga & Rodriguez-Sosa, 2002; Strauss & Dircksen, 2010, see Tomioka et al., 2012 and Fanjul-Moles, 2014 for a review).

In non-mammalian vertebrates, self-synchronizing peripherical oscillators (independent of the central time-keeper) have been identified only in certain districts such as the pineal gland, the retina and the hypothalamus. For instance, studies conducted on the frog *Xenopus laevis* revealed that its retinal pace-makers driving the rhythmical synthesis of melatonin works independently of the entrainment provided by the central oscillator present in the brain (Cahill & Besharse, 1993).

On the contrary, oscillators occurring in peripherical districts of mammalians are not autonomous and they are generally synchronized by a master clock localized in the suprachiasmatic nucleus of the hypothalamus in turn entrained through retinal photoreceptors to the external L:D cycle (Ralph et al., 1990; Klein et al., 1991; Reppert & Weaver, 2002; Schibler & Sassone-Corsi, 2002; see Yamazaky et al., 2000 for a review).

However, recent investigations provided evidence for the existence of peripherical self-sustaining time-keepers also in mammalians (Nagoshi et al., 2004; Yoo et al., 2004; Welsh et al., 2004).

2.2.2.1 Antennal time-keepers in arthropods: an overview

Although in crustaceans antennae can fulfil even other functions (swimming, attachment to host or substratum, feeding and mating behaviour), these appendages are mainly sensory structures. Sensilla, consisting of bipolar neurons (with sensory dendrites and axons projecting to the central nervous system), are distributed all along the limb. In particular, the flagellum is mainly characterized by olfactory neurons. The modulation of olfactory responses acquires an ecological significance in several species since behaviours such as predator avoidance, foraging and mating rely on the perception of specific olfactory stimuli. The circadian responsiveness to odorous substances is regulated by time-keepers localized in these appendages. In fact, investigations conducted in insects, like *Drosophila melanogaster* (Plautz et al., 1997; Krishnan et al., 1999, 2008; Tanoue et al., 2004), *Leucophaea maderae* (Page & Koelling, 2003; Saifullah & Page, 2009) and the moth *Spodoptera littoralis* (Merlin et al., 2007) revealed the existence of antennal pace-makers governing the rhythms of olfactory sensitivity exhibited by these species.

Instead, in *Danaus plexippus* it has been recently shown that antennal time-keepers are responsible for the compensation of the apparent movement of the sun and thus for the functioning of the sun compass mechanism. Indeed, individuals with antennae surgically removed were unable to orientate correctly (Merlin et al., 2009). These oscillators are independent of the central pace-maker and directly synchronized by the external photoperiod since the expression of canonical clock genes undergoes circadian oscillations even in explanted appendages entrained by an artificial L:D cycle. However, a central integration of the information from each antenna is fundamental for a correct time-compensated solar orientation (Guerra et al., 2012).

2.2.1 Endogenous oscillators in *T. saltator*

As previously illustrated, *T. saltator* exhibits nocturnal locomotor activity whereas during the day it remains buried into the belt of moist sand of the beach (Williamson, 1951; Pardi & Papi, 1953; Geppetti & Tongiorgi, 1967a, b; Scapini et al., 1992). Bregazzi and Naylor (1972) first demonstrated that this rhythm was in fact controlled by an endogenous oscillator. Indeed, they showed its persistence with a circadian periodicity under constant conditions (both DD and LL) and its independence of the external temperature. Moreover, these authors identified the L:D cycle as the main entrainment factor of the rhythm (although they suggested also the existence of social synchronization). Later investigations confirmed the role of the L:D cycle as a Zeitgeber entraining the activity rhythm of sandhoppers (Williams, 1980 a, b; Edward & Naylor, 1987) and in particular, it has been revealed a direct correspondence between the phase of the rhythm and the time of dawn (Williams, 1980c). Even further evidence for the mutual synchronization of the activity rhythm of this species have been provided (Nardi et al., 2003; Scapini et al., 2005).

Recent studies revealed a high intra-individual variability in the free-running period exhibited by sandhoppers (Nardi et al., 2003). It has been also observed that τ undergoes seasonal variations since in spring-summer it is closer to 24 h than in the rest of the year. Moreover, in the colder periods of the year the percentage of arrhythmic individuals decreases. These phenomena have been interpreted by Nardi et al. as ecological adaptions to minimize harmful effects of high temperature and low humidity conditions potentially occurring daytime over spring-summer.

Even the compensatory mechanism underlying sun compass-based orientation is driven by an endogenous pace-maker. In fact, sandhoppers released under the sun after their protract exposure to DD conditions performed directional choices in agreement with their escape direction (of course exhibiting small deflections with respect the expected direction due to the lack of light-entrainment) (Ercolini, 1960). Moreover, it has been shown that this timing mechanism works throughout the 24 h period. Indeed, individuals subjected to an L:D cycle inverted with respect the natural one, when released during their subjective night under the sun, orientated according to a “nocturnal” path of the astronomical body inverted compared to the “diurnal” one (i.e. from West to East passing through South, *Talitrus* model) (see Pardi, 1954b). Further investigations (Ugolini et al., 2002) demonstrated also a direct correspondence between the speed of the chronometric mechanism and the number of hours of light (out of a period of 24 h) sandhoppers had been exposed.

Some authors (Edward & Naylor, 1987) observed slight differences in the free-running periods of the orientation rhythm with respect that of locomotor activity suggesting that in this species different oscillators were implied in their regulation. However, recent investigations (Scapini et al., 2005; Ugolini et al., 2007) clarified that the same time-keeper underlies both the sun compass system and the locomotor activity rhythm. Indeed, sandhoppers kept in individual chambers (to avoid mutual synchronization) under constant conditions, preventing entrainment by the L:D cycle, exhibited in any case both time-compensated solar orientation and locomotor activity rhythm concordant each other, in accordance with the same free-running period (Scapini et al., 2005). Moreover, it has been revealed (Ugolini et al., 2007b) that the exposure to phase-shifted L:D cycles affect not only the activity rhythm of sandhoppers (i.e. animals were mainly active during their subjective night) but also their solar orientation since individuals orientated accordingly to their subjective time.

Evidence for clock-controlled lunar orientation were also provided (Papi & Pardi, 1959; Enright, 1972). In fact, sandhoppers were able to assume a correct compass direction even after being subjected to DD conditions. However, the capability of this species to rely on the moon was call into question mainly because of the theroretical difficulties animals meet in the correct use of this factor as a cue (see above) (Enright, 1961, 1972; Craig, 1971; see also Wallraff, 1981). Only recently (Ugolini et al., 1999b), the debate was clarified and the existence of a moon compass mechanism in *T. saltator* was fully confirmed. Moreover, further studies (Ugolini et al., 2007a) suggested that the chronometric mechanism underlying moon-based orientation exhibits circalunar periodicity and works throughout the lunar month. Indeed, in tests carried out during the new moon phase (when the moon is not visible) by simulating the astronomical body with an artificial source of illumination individuals orientated effectively to the fake moon and directed toward their escape direction.

Studies conducted to establish the relationship between the time-keepers underlying the sun and the moon compass mechanisms revealed the existence of discrete oscillators responsible for their regulation (Ugolini et al., 1999b). In fact, whereas individuals subjected to phase-shifted L:D cycles orientated to the sun accordingly to their subjective time, their lunar orientation was not influenced by this treatment and they directed correctly toward their escape direction. On the contrary, sandhoppers exposed to phase-shifted lunar cycles orientated to the moon in accordance with the artificial cycle they were subjected to and their solar orientation was not affected (Ugolini et al., 1999b). The existence of separate time-keepers responsible for the functioning of the two compass systems was also shown in further investigations (Ugolini et al., 2012) in which researchers confirmed that the exposure to phase-shifted L:D cycles affects only the mechanism of sun compass and not that underlying lunar orientation. Indeed, sandhoppers exposed to an L:D cycle inverted with respect the natural one when released under the sun (during their subjective night) exhibited directional choices in accordance with the compensation of the sun at night (*Talitrus* model, Pardi 1954b); instead when tested under the moon (during their subjective day) they orientated correctly toward their escape direction (i.e. their lunar orientation were not influenced by the experimental treatment).

However, results obtained by Ugolini et al. (1999b, 2012) are in contrast with those achieved in other investigations (Meschini et al., 2008). Indeed, in their work Meschini et al. revealed that both L:D and lunar phase-shifted cycles affected the lunar orientation of sandhoppers causing correspondent deflections in their directional choices. Therefore, without excluding the involvement of a time-keeper exhibiting circalunar periodicity in time-compensated moon-based orientation, they rejected the hypothesis of the independence of the two compass mechanisms. In particular, authors speculated on a three components-controlled rhythm. Indeed, they supposed that the moon compass mechanism could be regulated by three discrete oscillators exhibiting circadian, circalunidian (period ~ 24.8 h, lunar day) and circalunar periodicities respectively (“three rhythms hypothesis”).

3 Purposes of the work

Taking into account the argument illustrated above, the aims of my work are:

- to deepen our knowledge on the use of celestial factors by *T. saltator*. In particular, to assess its ability to rely on the celestial spectral pattern;
- to investigate the putative regionalization of its visual capabilities;
- to assess the optical and functional features of its compound eye;
- to test the possible involvement of the antennae in solar and/or lunar orientation;
- to evaluate the anatomical localisation of the time-keepers driving sun and moon chronometric compensations;
- to establish the existence of a moon compass-based orientation in syntopic species with *T. saltator* (the isopod *T. europaeus*).

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CHAPTER 2

THE CELESTIAL GRADIENTS AS COMPASS ORIENTING FACTORS IN *TALITRUS SALTATOR* (CRUSTACEA, AMPHIPODA)

Results reported in this chapter have been obtained in collaboration with Dr. Luca Mercatelli (National Institute of Optics, National Research Council of Italy, CNR-INO).

Introduction

The supralittoral sandhopper *Talitrus saltator* (Montagu, 1808) is one of the best-known biological model among arthropods for studies of celestial orientation. In fact, since the Fifties many investigations have demonstrated its capability to rely on both the sun and the moon to return to the belt of damp sand (in which animals remain buried during the day) following the shortest route (i.e. the sea-land axis of the shore) (Papi & Pardi, 1953, 1954, 1959; Pardi & Papi, 1953; Ugolini et al., 1999, 2007). Of course, the use of these astronomical bodies requires endogenous chronometric mechanisms accountable for compensating their azimuthal variations and maintain a constant effective direction throughout the 24 h period.

However, the sun and the moon are not the sole celestial orienting factors used by animals. Indeed, the scattering of the sunlight occurring during its passage through the atmosphere defines, besides a polarization pattern, profiles of luminance and spectral distribution across the sky (see Waterman, 1981 for a review). To perceive and rely on these cues, providing directional information concordant with those furnished by the sun, allows individuals to properly orientate even when the astronomical body is not directly visible.

Although celestial polarization constitutes a reliable orienting reference for many arthropods (see Waterman, 1981; Horvàth &Varju, 2004 for a review), it has been shown that sandhoppers can not rely on this factor in their zonal recovery (Pardi & Papi, 1953; Ugolini et al., 2013).

Verheijen (1978) first suggested that the skylight luminance gradient, defined by light intensity variations along the solar meridian, could help animals orientate but since then scarce investigations have been conducted to assess its use among arthropods (Wehner, 1997, Ugolini et al., 2009; el Jundi et al., 2014a). To date, *T. saltator* is the sole species known able to obtain directional information from this reference sufficient to direct correctly toward its preferred zone (Ugolini et al., 2009). On the contrary, the navigational system of the desert ant *Cataglyphis bicolor* is disrupted when allowed to orientate using only this factor; in fact, individuals lose their ability to assume a correct homeward direction and perform phototactic responses toward the brighter region of the sky (Wehner, 1997). Instead, the dung beetle *Scarabaeus lamarki* (not performing time-compensated orientation) can rely on this reference but only in combination with the polarization pattern (el Jundi et al., 2014a).

However, the celestial light intensity gradient varies depending on the degree of cloud cover across the sky and the eventual capability of sandhoppers to perceive and rely on luminance profiles attenuated with respect that occurring in clear sky conditions has never been assessed.

Any investigation has even been conducted to evaluate the putative ability of *T. saltator* to use the celestial spectral gradient as an orienting cue in its zonal recovery. This factor 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 one ("antisolar" hemidome) defining a continuum along the solar meridian. Some arthropods, like the honey bee *Apis mellifera* (Brines & Gould, 1979; Edrich et al., 1979; Rossel & Whener, 1984), the desert ant *Cataglyphis bicolor* (Wehner, 1997) and the dung beetle *Scarabaeus lamarcki* (el Jundi et al., 2015a, b), are known to discriminate the sun and the anti-sun direction and perform effective directional choices relying on celestial spectral stimuli characterized by longer and shorter wavelengths respectively.

Recent studies (Ugolini et al., 2010) revealed the sensitivity of *T. saltator* to UV-blue (λ = 390-450 nm) and green (λ = 500-550 nm) light allowing us to speculate on the capability of this species to obtain compass information from the spectral pattern of the sky as well.

Therefore, the aims of this work are 1) to evaluate the ability of *T. saltator* to detect and use skylight intensity profiles attenuated with respected that occurring in conditions of clear sky and 2) to assess the competence of this species to rely on the celestial spectral gradient in its zonal recovery.

Material and methods

Tests were conducted by releasing adult individuals of *T. saltator*, collected 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°) over spring-summer 2013-2017, within 20 days from their capture.

After their collection, animals were kept in the laboratory in Plexiglas boxes containing wet sand at room temperature (25°C) and under a light:dark (L:D) cycle = 12:12 in phase with the natural photoperiod (sunrise = 0600 hours, sunset = 1800 hours). Food (universal dried food for fish, SERA® Vipan, Heisenberg, Germany) was available *ad libitum*.

Experiments were carried out in Florence (43°46’45"N, 11°14’46"E) from 0830 hours to 0930 hours (antimeridian tests), from 1130 hours to 1230 hours (meridian tests) and from 1530 hours to 1630 hours (pomeridian tests).

Sandhoppers were released in a confined environment using the typical device for experiments of orientation in this species (Pardi & Papi, 1953; Ugolini & Macchi, 1988) (Fig. 1). It consisted on a tripod supporting a horizontal transparent plate (diameter = 30 cm) with a Plexiglas bowl (diameter = 20 cm) placed on into which sandhoppers were released. A goniometer was set under the bowl to measure individuals' directional choices. The vision of the surrounding landscape was prevented with a white cylindrical Plexiglas screen (diameter = 30 cm, height = 6 cm).

Fig. 1. Experimental device used in behavioural tests.

Groups of 10-12 individuals were tested at a time since it was demonstrated their non-reciprocal influence in performing directional choices (Scapini et al., 1981). 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 from freeze-framed images taken by using a video-camera placed under the apparatus.

Animals were dehydrated for a few minutes before being tested to motivate them to rapidly orientate seaward (265°).

Releases were carried out in the laboratory by showing individuals artificial skies reproducing luminance and spectral profiles whose "solar meridian" coincided in any cases with the East-West axis. Directions registered were then translated taking into account the orientation of the natural gradients at the times of tests.

The natural luminance and spectral patterns were characterized through spectroradiometric measurement (spectroradiometer mod. CS1000, Minolta, Osaka, Japan) conducted in conditions of clear sky at intervals of 15 degrees along the solar meridian (zenithal distance of the sun = 45°) (Fig. 2A-B).

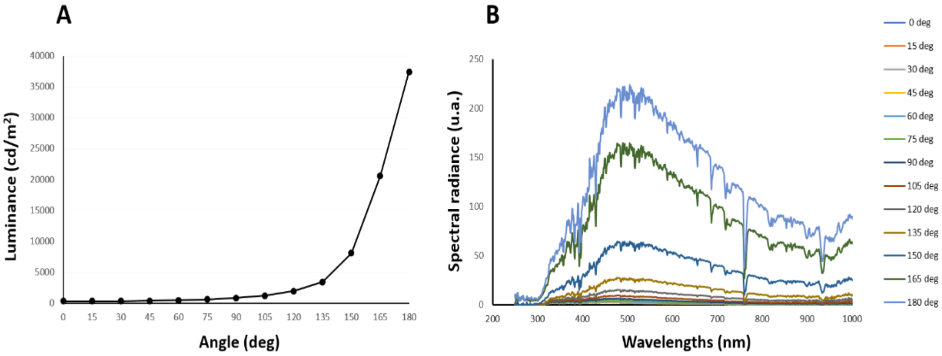

Fig. 2. Luminance (A) and spectral (B) natural profiles measured in clear sky conditions at intervals of 15 ° (sun zenithal distance = 45°).

Artificial luminance profiles

Artificial profiles of light intensity were designed using the software Matlab® (MATrix LABoratory) and projected onto a circular opaline Plexiglas plate (diameter = 30 cm), placed over the bowl containing the animals, by using a video-projector provided of a halogen lamp uhp 200w (mod. LC4341, Philips, Made in Austria).

A profile reproducing the natural gradient of luminance occurring in conditions of clear sky (Fig. 3A) was realized by assigning to each numerical value of light intensity (measured along the solar meridian in natural conditions) a shade of grey.

The artificial sky was then characterised through radiometric measurements conducted at intervals of 10 degrees by placing the spectroradiometer (mod. CS1000, Minolta, Osaka, Japan) on a stand placed in the position generally occupied by the bowl. Variations of light intensity revealed along the solar meridian of the artificial sky are reported in Fig. 3B.

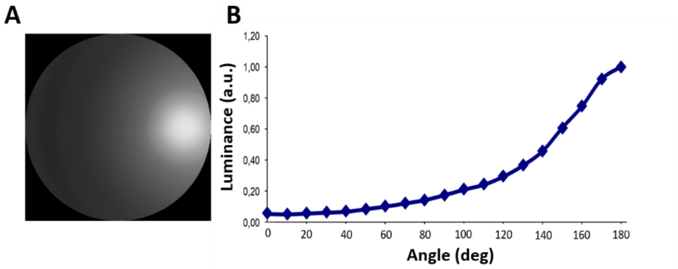

Fig. 3. Artificial celestial profile corresponding to the luminance pattern occurring in clear sky conditions. (A) image used as artificial sky, (B) light intensity variations along the solar meridian (blue curve).

Moreover, celestial profiles attenuated with respect the gradient present in conditions of clear sky were designed by reducing light intensity variations along the solar meridian. In particular, luminance patterns attenuated by 40% (Fig. 4A) and 75% (Fig. 5A) were elaborated. Variations of light intensity revealed through radiometric measurements along the solar meridian are reported in Fig .4B and 5B respectively.

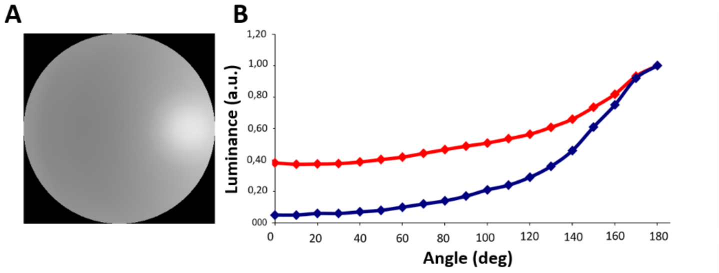

Fig. 4. Artificial celestial profile attenuated by 40% with respect the luminance gradient occurring in clear sky conditions. (A) image used as artificial sky, (B) reduction of the light intensity variations (red curve) with respect the natural pattern (blue curve).

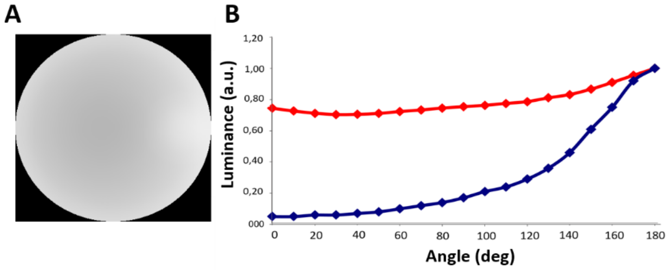

Fig. 5. Artificial celestial profile attenuated by 75% with respect the luminance gradient occurring in clear sky conditions. (A) image used as artificial sky and (B) reduction of the light intensity variations (red curve) with respect the natural pattern (blue curve).

Artificial spectral profiles

In order to avoid any interference in sandhoppers' directional choices, spectral profiles were designed by minimizing light intensity variations along the solar meridian. Of course, their elaboration was conducted by taking into account the spectral sensitivity of this species (Fig. 6).

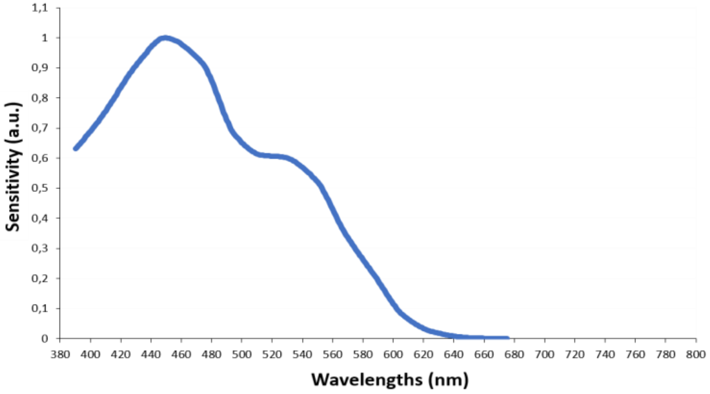

Fig. 6. Spectral sensitivity of *T. saltator*.

Also in this case, artificial skies were elaborated by using the software Matlab® and projected over the Plexiglas opaline plate.

Spectral profiles divided into sections each corresponding to a colour (green and blue) combination defining a gradient from "pure green" (corresponding to the "solar" hemidome) to "pure blue" (corresponding to the "antisolar" hemidome) (Fig. 7A-B) were first realised. In the artificial sky reported in Fig. 7B red wavelengths (not detected by sandhoppers) were homogenously added to reduce the overall luminance perceived.

Then, spectral profiles simulating the natural blue gradient of the sky divided into 5 and 7 sections (Fig. 7C and 7D respectively) each corresponding to a different tonality of blue were elaborated.

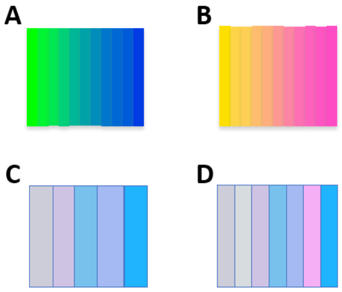

Fig. 7. Artificial green-blue spectral profiles designed by using the software Matlab®**.** (A) blue-green profile, (B) blue-green profile, red wavelengths added, (C) profile representing the natural blue gradient of the sky divided into 5 sections and (D) profile representing the natural blue gradient of the sky divided into 7 sections.

Moreover, the apparatus described above was modified by placing opaline Plexiglas domes (diameter = 30 cm and 80 cm) over the bowl containing the animals. The internal surface of the domes (the artificial skies) was illuminated by a fiber-optic illuminator (Schott KL1500). The end of the fiber bundle (diameter = 8 mm) was housed in a tube at the center of the bowl so that the light source was as close as possible to the center of the dome (Fig. 8).

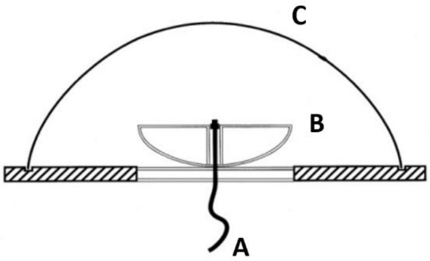

Fig. 8. Schematic representation of the device modified by using a fiber-optic illuminator lighting the internal surface of opaline domes. (A) fiber bundle, (B) bowl containing sandhoppers and (C) Plexiglas opaline dome.

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 a hemicycle of the fiber bundle. Light intensity variations along the solar meridian were minimized by placing two neutral density grey filters (no 298 0.15, transmittance = 69,3% and no 209 0.3, transmittance = 51.2%, Illuminoservice, Bologna, Italy) superimposed on the opposite hemicycle.

Spectroradiometric measurements (spectroradiometer mod. CS1000, Minolta, Osaka, Japan) were conducted inside each dome along the solar meridian at intervals of 10 degrees. In Fig. 9A and 9C there are reported the luminance profiles recorded inside the 30 cm diameter dome and the 80 cm diameter dome respectively. In both cases, light intensity variations were considered negligible since not appropriate to provide sandhoppers directional information. Moreover, spectral data were elaborated using the software Matlab® to assess the level of stimulation of the two discrete photoreceptors of sandhoppers at each interval of measurement (Fig. 9B and 9D).

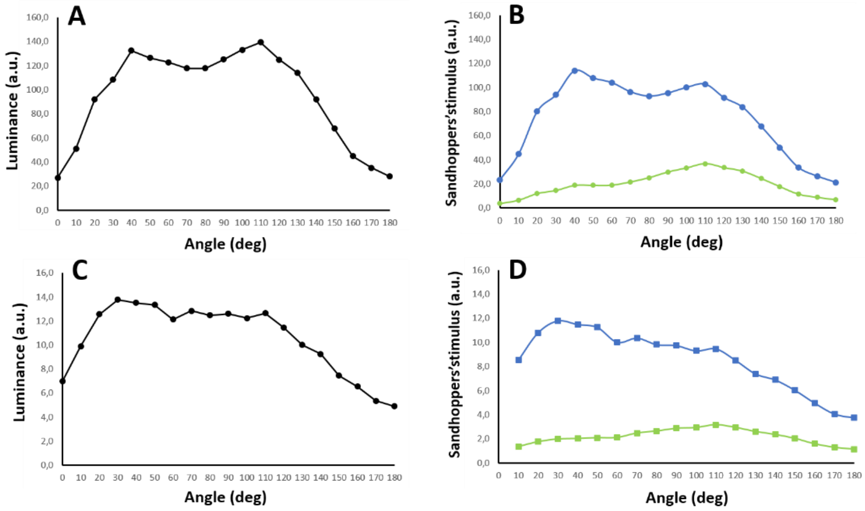

Fig. 9. Spectroradiometric characterization of the domes (A) 30 cm diameter dome light intensity profile, (B) level of photoreceptors stimulation inside the 30 cm diameter dome (blue curve: UV-blue sensitive pigment, green curve: green sensitive pigment), (C) 80 cm diameter dome light intensity profile and (D) level of photoreceptors stimulation inside the 80 cm diameter dome (blue curve: UV-blue sensitive pigment, green curve: green sensitive pigment).

Statistical analysis

Data were analysed 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).

Results

Artificial luminance profiles

Sandhoppers released under the artificial sky reproducing the light intensity profile present in conditions of clear 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. 10A).

Instead, when tested by using artificial luminance patterns attenuated by 40% and 75% compared to that occurring in conditions of clear sky, individuals did not exhibit directional choices in accordance with their escape direction; in fact, they showed in both cases phototactic responses toward the brighter region of the sky (Fig. 10B-C).

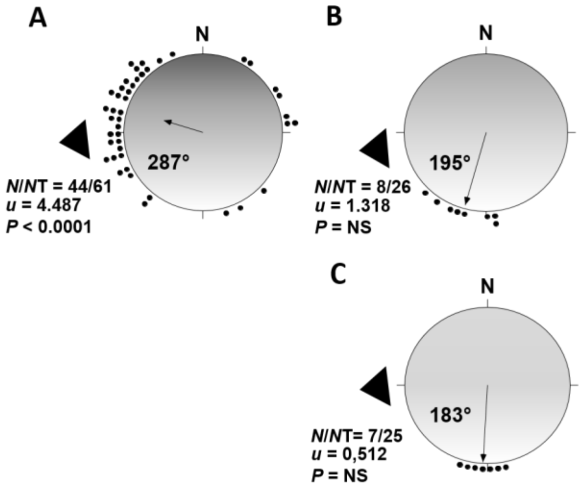

Fig. 10. Releases under artificial luminance profiles. (A) sandhoppers released under the artificial luminance pattern corresponding to that occurring in conditions of clear sky, (B) sandhoppers released under the artificial pattern attenuated by 40% and (C) sandhoppers released under the pattern attenuated by 75%. N, North, black arrows, mean vector and angle (the length of the mean vector ranges from 0 to 1 = radius of the circle), black dots, sandhoppers' directions (each dot corresponds to the direction of one individual); black triangles outside the distributions, seaward direction of animals' home beach. *N*/*N*T, number of radially oriented sandhoppers (*N*) out of the total individuals tested (*N*T). The values of the V-test, *u*, with their probability level, *P*, are also given.

Artificial spectral profiles

Individuals tested by using artificial green-blue celestial profiles designed by Matlab® did not direct toward the seaward direction of their home beach. Indeed, when released under both the spectral profile without red wavelengths added (Fig. 11A) and the spectral profile with red wavelengths added (Fig. 11B) sandhoppers orientated toward the blue ("antisolar") hemicycle of the sky.

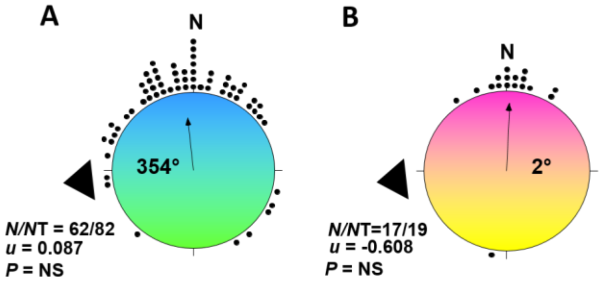

Fig. 11. Releases under artificial green-blue spectral profiles. (A) sandhoppers released under the spectral pattern without red wavelengths added and (B) sandhoppers released under the spectral pattern with red wavelengths added. See Fig. 10 for further information.

Even in trials carried out under the spectral profiles simulating the natural blue gradient of the sky sandhoppers do not perform directional choices in accordance with their escape direction and constantly orientated toward the blue hemidome of the sky (Fig. 12A-B).

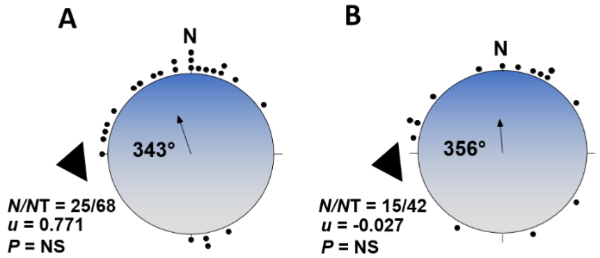

Fig. 12. Releases under artificial spectral profiles corresponding to the natural blue gradient of the sky. (A) sandhoppers released under spectral pattern divided into five sections and (B) sandhoppers released under spectral pattern divided into seven sections. See Fig. 10 for further information.

On the contrary, when tested under the Plexiglas domes whose internal surface was lightened by the fiber-optic illuminator individuals orientated correctly toward the seaward direction of their home beach. In both antimeridian (Fig. 13A) and pomeridian (Fig. 13B) releases carried out under the 30 cm diameter dome the mean orientation of sandhoppers was in good agreement with the expected direction with a deflection of only 22° and 18° respectively. Similarly, the mean orientation of individuals released under the 80 cm diameter dome (Fig. 13C-E) was in each case concordant with their escape direction (deflection of the mean resultant vectors: antimeridian tests = 27°; meridian tests = 31°; pomeridian tests = 32°).

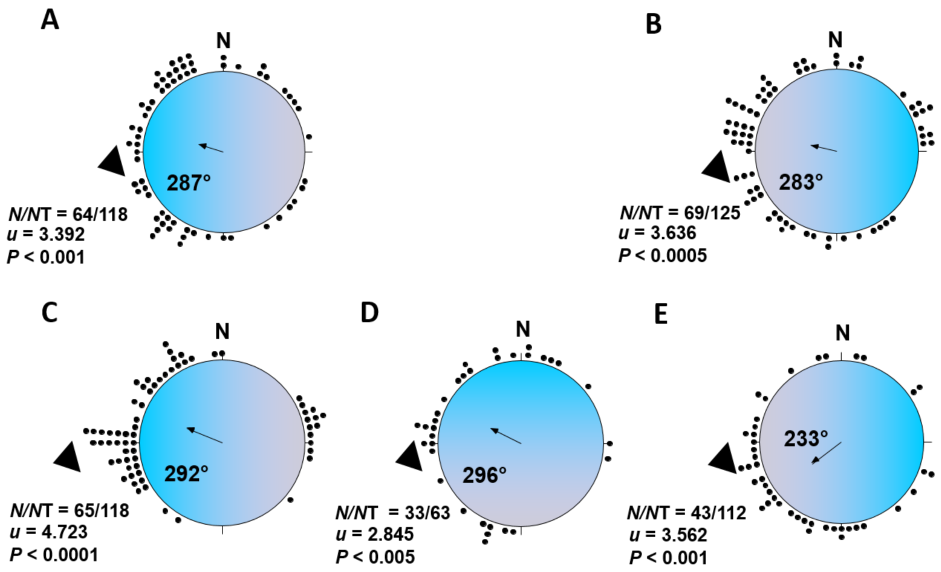

Fig. 13. Releases under opaline Plexiglas domes with fibre-optic illuminator lighting their internal surface. (A) sandhoppers tested under the 30 cm diameter dome (antimeridian tests), (B) sandhoppers tested under the 30 cm diameter dome (pomeridian tests), (C) sandhoppers tested under the 80 cm diameter dome (antimeridian tests), (D) sandhoppers tested under the 80 cm diameter dome (meridian tests) and (E) sandhoppers tested under the 80 cm diameter dome (pomeridian tests). See Fig. 11 for further information.

Conclusions

This work clearly confirms the capability of *T. saltator* to use the skylight pattern of luminance as a compass cue in its zonal orientation. In fact, animals released under the artificial sky reproducing the gradient occurring in conditions of clear sky orientated correctly toward their escape (seaward) direction. On the contrary, when released under artificial luminance profiles attenuated at least by 40% individuals did not perform effective directional choices.

As previously stated, the celestial intensity pattern could be used even when the sun is not directly visible; of course, in this case the luminance variations along the solar meridian are reduced (dependently of the degree of cloud cover) with respect those occurring in conditions of clear sky. Therefore, to perceive attenuated profiles is crucial to obtain effective ecological advantages from this factor. Results suggest that it exists a minimum threshold of the gradient detectable by *T. saltator* and thus, in natural conditions, it can not be always used as an orienting reference. Further investigations could assess the least variations in light intensity along the solar meridian sufficient to provide sandhoppers compass information.

In tests conducted by releasing individuals under the artificial spectral profiles reproduced inside the domes, first evidence for the use of the spectral gradient of the sky as a compass cue by *T. saltator* was obtained. In fact, individuals performed time-compensated directional choices and orientated correctly toward their escape direction independently of the time of the test (and thus of the disposition of the spectral profile across the artificial sky). However, further analyses could be addressed to refine the experimental procedures in order to minimize the light intensity variations perceived by sandhoppers across the artificial skies pointed out by spectro-radiometric measurements (see Fig. 10A and 10C).

On the contrary, when released under the spectral profiles designed with Matlab® individuals did not exhibit chronometrically-compensated orientation but constantly directed toward the blue ("antisolar") celestial hemicycle. These results suggest that the absence of a spectral continuum along the solar meridian in profiles divided into sections or their excessive dissimilarity with respect the natural pattern could prevent their proper recognition and their use as compass orienting cues by this species.

Future studies could assess even the eventual capability of *T. saltator* to obtain effective compass information also from single celestial spectral stimuli, similarly to other arthropods (Brines & Gould, 1979; Edrich et al., 1979; Rossel & Wehner, 1984; Wehner, 1992; el Jundi et al., 2015a, b).

Neuroanatomical investigations conducted in some species of insects revealed that information provided by the celestial polarization pattern and intensity/spectral profiles, detected by discrete regions of the eye, are integrated into the central nervous system to generate a consistent compass signal (el Jundi et al., 2014b). Although *T. saltator* does not rely on the celestial polarization in its zonal recovery (Pardi & Papi, 1953; Ugolini et al., 2013), to perceive and use multiple celestial cues could make more reliable its internal compass system and thus render more efficient its time-compensated orientation.

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CHAPTER 3

REGIONALIZATION OF THE VISUAL CAPABILITIES OF *TALITRUS SALTATOR* (CRUSTACEA, AMPHIPODA)

Introduction

The supralittoral sandhopper *Talitrus saltator* (Montagu, 1808) to return as quick as possible to the belt of damp sand in which it lives buried during the day orientates along the sea-land axis of the beach, perpendicular to the shoreline (characteristic for each population dependently on the orientation of the shore). This species to avoid high temperature and low humidity conditions, that can rapidly lead animals to death, evolved nocturnal habits. However, disturbing factors, such as predators, strong winds or changes in the sea level, can cause active or passive displacements of sandhoppers from their preferred zone also during the day. In any case, animals orientate seaward when exposed to dry conditions whereas submersion motivates them to direct landward.

In its zonal recovery, *T. saltator* is known to rely on several celestial cues; of course, their use requires continuous adjustments to account for their temporal azimuthal variations and maintain a constant effective direction. Evidence for time-compensated solar and lunar orientation has been provided since the Fifties (Papi & Pardi, 1953, 1954, 1959; Pardi & Papi, 1953; see Pardi & Ercolini, 1986 for a review). Recently (Ugolini et al., 2009), it has been shown that also the skylight intensity gradient constitutes a compass reference for this species.

Another celestial factor perceived and used as a compass cue to orientate effectively by many arthropods like *Apis mellifera* (von Frisch, 1949), *Gryllus campestris* (Brunner & Labhart, 1987), *Cataglyphis bicolor* (Duelli & Wehner, 1973) and *Danaus plexippus* (Reppert et al., 2004) is the polarization pattern due to the atmospheric sunlight scattering and concordant with the position of the sun (Menzel, 1975; Brines & Gould, 1982; Wehner, 1989; Tovee, 1995; Eguchi, 1999). Moreover, even other species such as the dung beetle *Scarabaeus lamarki* (el Jundi et al., 2014), although not performing time-compensated orientation, can obtain orienting information from celestial polarization.

Several investigations revealed that animals to acquire directional information mainly use polarized light in the UV since less affected than longer wavelengths by adverse atmospheric conditions (Duelli & Wenher, 1973; Menzel & Snyder, 1974; Waterman, 1981; Brines & Gould, 1982; Stalleicken et al., 2005).

The perception of polarized light involves ommatidia localized in the dorsal margin of the compound eye (Dorsal Rim Area, DRA) exhibiting specific morphological adaptions to detect the e-vectors pattern (see Labhart & Meyer, 1999 for a review). In some species, such as *Apis mellifera* (Wehner & Strasser, 1985), *Gryllus campestris* (Labhart et al., 1984), *Cataglyphis bicolor* (Duelli & Wehner, 1973; Wehner, 1992), it has been shown a spatial distribution of their visual pigments. In fact, UV-sensitive cells (also responsible for polarized light detection) are localized in the DRA, whereas those sensitive to longer wavelengths are mainly present in the ventral region of the eye.

The use of the celestial polarization pattern as a compass cue by *T. saltator* has been investigated but, although preliminary evidence for its capability to detect polarized light, it seems that this factor does not provide sandhoppers compass information (Pardi & Papi, 1953). In fact, animals released in a confined environment under a polarizing-filter exhibited a general difficulty to perform directional choices. Moreover, they showed stress signals like the formation of clusters (i.e. each animal tried to hide under the others to avoid dehydration). However, individuals varied their clustering arrangement on the basis the orientation of the filter concentrating in the quadrants including the expected direction according to the polarizer positioning. Similar investigations conducted lately (Ugolini et al., 2013) revealed that sandhoppers lost their capability to orientate only when the vision of the sun was prevented. Even in this case, it was observed a tendency to form clusters whose arrangement changed correspondingly to the polarizing-filter orientation.

Over the years, evidence for the sensitivity of *T. saltator* to UV-blue wavelengths was provided (Ercolini & Scapini, 1976; Laffort, 1992; Mezzetti & Scapini, 1995; Ugolini et al., 1996). It was also shown that a celestial factor in the UV-blue range was fundamental for the proper time-compensated solar orientation of this species since its ability to orientate under the sun was disrupted when the perception of wavelengths shorter than 450 nm was prevented. It was supposed that this cue was the celestial polarization pattern (Ugolini et al., 1993, 1996). Recently, behavioural tests and electrophysiological recordings confirmed clearly the sensitivity of this species to UV-blue (λ = 390-450 nm) and green (λ = 500-550 nm) light and it was demonstrated that discrete photoreceptors are involved in the perception of the two ranges of wavelengths (Ugolini et al., 2010).

Moreover, it has been shown that sandhoppers can detect polarized light in the blue wavelength range since in tests of binary choice they significantly directed toward a linearly polarized light source rather than a non-polarized one when irradiated with blue light (λ = 435 nm). Instead, animals did not perform any preference between polarized and non-polarized light when tested with white or green sources of illumination (Ugolini et al., 2013). Skylight polarization could be involved in increasing the intensity and chromatic contrast across the sky thus facilitating the perception of other celestial factors but to date, its actual role in the zonal recovery of sandhoppers remains unknown.

Although the existence of a DRA within the eye of *T. saltator* has never been investigated, the aim of this work is to assess the eventual regionalization of the visual capabilities in this species, as well as shown in other arthropods exhibiting specialized adaptions of their dorsal ommatidia suitable to detect polarized light.

Material and methods

Behavioural tests were conducted by releasing adult individuals of *T. saltator*, collected 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°) over spring-summer 2016, within 14 days from their capture.

After their collection, animals were transported to the laboratory and kept in Plexiglas boxes containing damp sand. Food (universal dried food for fish, SERA, Vipan, Heisemberg, Germany) was available *ad libitum*.

Sandhoppers were maintained under an L:D = 12:12 cycle in phase with the natural photoperiod (sunrise = 0600 hours, sunset =1800 hours) and at ambient temperature (25 °C).

Experiments were carried out in a confined environment by using a device similar to that described by Ugolini and Macchi (1988) (Fig. 1). It consisted on a tripod supporting a horizontal transparent plate (diameter = 30 cm) with a Plexiglas bowl (diameter = 20 cm) placed on where sandhoppers were released. A goniometer was set under the bowl to detect the directions assumed by individuals. The vision of the surrounding landscape was prevented by using an opaline Plexiglas screen (height = 7 cm).

Fig.1. Experimental device used for behavioural tests

Groups of 10-15 sandhoppers were released into the bowl at a time since it has been demonstrated their non-reciprocal influence in performing directional choices (Scapini et al., 1981). A single direction (error ± 2.5°) for each radially-oriented individual (with the head pointed toward the outside of the bowl and with the longitudinal axis of the body oriented no more than ± 45° from the radius of the bowl) was recorded by using a video-camera placed under the apparatus. In each trial, the number of not-radially oriented individuals was also registered.

Experiments were conducted between 1200 and 1300 hours; sandhoppers were dehydrated for a few minutes before being tested to motivate them to orientate seaward.

Releases were carried out both in conditions of natural sky visible and by placing the following gelatine filters (Spotlight, Milan, Italy) over the bowl containing the animals:

- grey filter (Medium Grey, no 210 Neutral Density): transmitting 23.5% of the total amount of light without modifying its spectral composition (Fig. 2A).
- CR39 filter blocking wavelengths shorter than 400 nm (UV light) (Fig. 2B).

- blue filter (Moonlight Blue, no 183): transmitting wavelengths between 320 nm and 600 nm (λ_{max} = 450 nm), transmittance = 73% (Fig. 2C).
- green filter (Leaf Green, no 121): transmitting wavelengths between 450 nm and 590 nm (λ_{max} = 500 nm), transmittance = 64% (Fig. 2D).

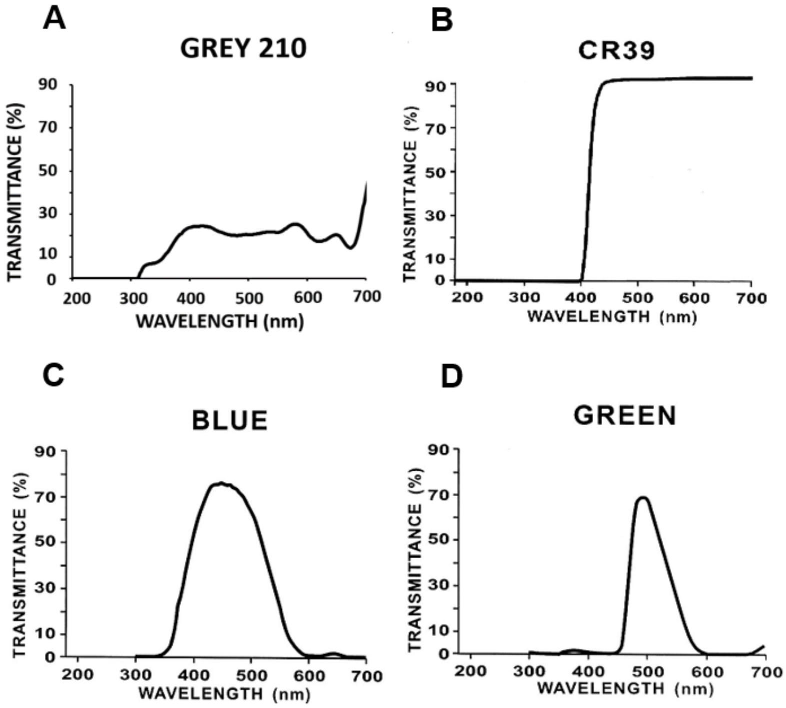

Fig. 2. Gelatine filters transmittance curves: (A) 210 ND grey filter, (B) CR39 filter, (C) Blue filter (Moonlight Blue, no 183) and (D) Green filter (Leaf Green, no 121).

Experiments were conducted both with the natural sun visible and with its vision prevented using a wood screen (40 X 40 cm).

To investigate the spatial distribution of the photoreceptors the following groups of individuals were released:

1) animals with eyes not painted;
2) animals with the 1/3 dorsal region of their eyes (corresponding to the Dorsal Rim Area of other arthropods) painted with black enamel (Rainbow, Maimeri, S.p.A., Mediglia, Milano, Italy) (Fig. 3A);
3) animals with their 2/3 ventral region of the eye painted (Fig. 3B).

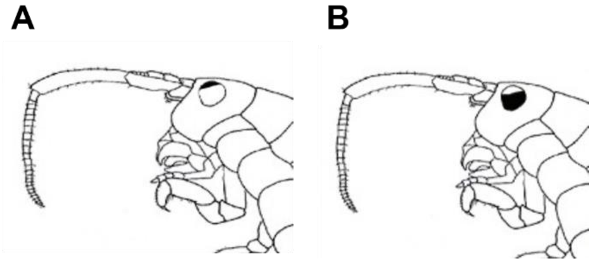

Fig. 3. Experimental treatments. A) 1/3 dorsal region painted. B) 2/3 ventral region painted.

Data were analysed according to the methods proposed by Batschelet (1981) for circular distributions. The length of the mean resultant vector and the mean angle were calculated. To establish whether the distributions differed statistically from uniformity the V-test was used (*P* < 0.05 at least).

Since the frequency of radially-oriented individuals is considered a good indicator to assess the difficulty of sandhoppers in their directional choices we conducted statistical comparisons between frequencies recorded in different trials using the G-test (*P* < 0.05 at least) (Zar, 1984).

Results

In tests conducted under the natural sky and the sun visible sandhoppers with their eyes not painted orientated significantly toward the seaward direction of their home beach (difference between the mean resultant vector and the expected direction = 26°) (Fig. 4A). Similarly, animals with either the 1/3 dorsal (Fig. 4B) or the 2/3 ventral (Fig. 4C) region of their eyes painted showed a mean orientation in accordance with the expected direction with only a modest deflection of the mean resultant vectors (7° and 17° respectively). However, statistically significant differences in the frequencies of radially-oriented individuals were observed between trials (*G* = 12.385, df = 2, *P* < 0.001, G test) (Fig. 5).

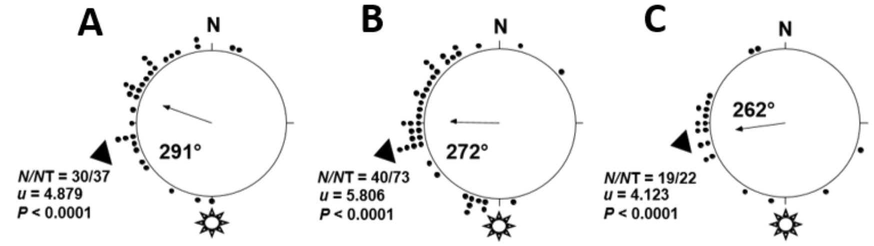

Fig. 4. Releases under the natural sky with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. N, North, black arrows, mean vector and angle (the length of the mean vector ranges from 0 to 1 = radius of the circle), black dots,

sandhoppers’ directions (each dot corresponds to the direction of one individual); black triangles outside the distributions, seaward direction of animals’ home beach. The symbol of the sun indicates the solar azimuth at the time of releases respectively. *N*/*N*T, number of radially oriented sandhoppers out of the total individuals tested. The values of the V-test, *u*, with their probability level, *P*, are also given.

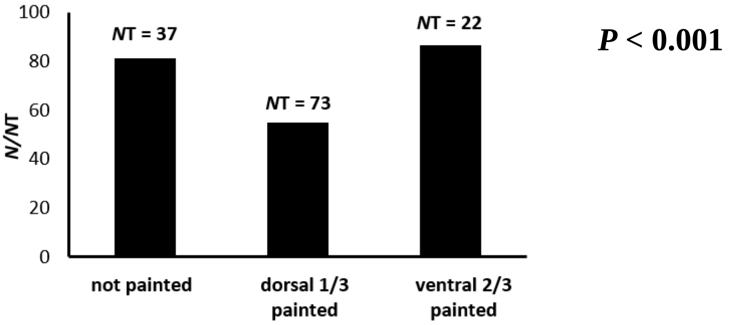

Fig. 5. Radially-oriented frequencies in releases carried out under the natural sky with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

When tested under the natural sky with the vision of the sun prevented not treated individuals (Fig. 6A) and sandhoppers with either the 1/3 dorsal (Fig. 6B) or the 2/3 ventral (Fig. 6C) region of their eyes painted orientated correctly toward the expected direction (deflection of the mean vectors from the expected seaward direction = 14°, 28°, 0° respectively). In this case, frequencies of radially-oriented individuals registered in releases of different groups of sandhoppers do not differ statistically each other ($G = 2.778$, df = 2, P = NS, G test) (Fig. 7).

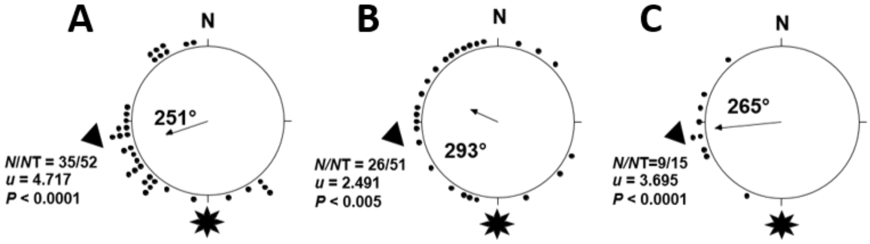

Fig. 6. Releases under the natural sky with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. The symbol of the black sun indicates the azimuth of the screened at the time of releases respectively. See Fig. 4. for further information.

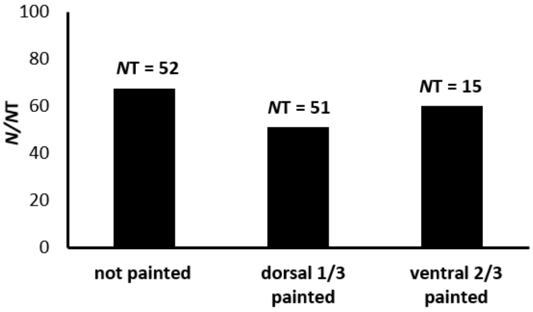

Fig. 7. Radially-oriented frequencies in releases carried out under the natural sky with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

In tests carried out under the grey filter with the sun visible sandhoppers with their eyes not painted orientated significantly toward the expected direction (deflection of the mean vector = 4°) (Fig. 8A). Even individuals subjected to experimental treatments performed directional choices in accordance with the seaward direction of their home beach. In fact, the mean resultant vectors obtained from releases of animals with either the 1/3 dorsal or the 2/3 ventral portion of their eyes painted were deflected by only 4° and 18° respectively (see Fig. 8B-C). Any statistically significant differences between trials in the frequencies of radially-oriented individuals were pointed out (*G* = 0.261, df = 2, *P* = NS, G test) (Fig. 9).

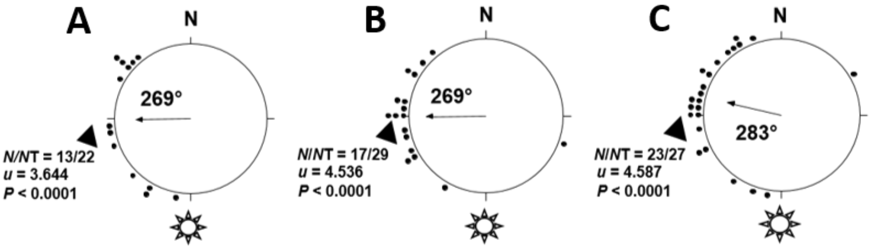

Fig. 8. Releases under the grey filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 4. for further information.

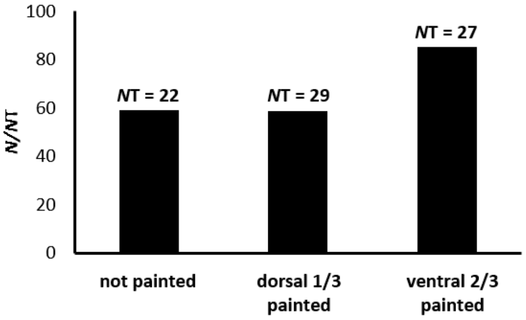

Fig. 9. Radially-oriented frequencies in releases carried out under the grey filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

In tests carried out under the grey filter and with the vision of the sun prevented each group of sandhoppers released performed a mean orientation in good agreement with their escape (seaward) direction. In any case the deflection of the mean resultant vector was modest (animals not treated = 1°, Fig. 10A; animals with the 1/3 dorsal region of their eyes painted = 15°, Fig. 10B; animals with the 2/3 ventral region of their eyes painted = 8°, Fig. 10C). Any significant difference between frequencies of radially-oriented individuals recorded in different trials was revealed (*G* = 4.446, df = 2, *P* = NS, G test) (Fig. 11).

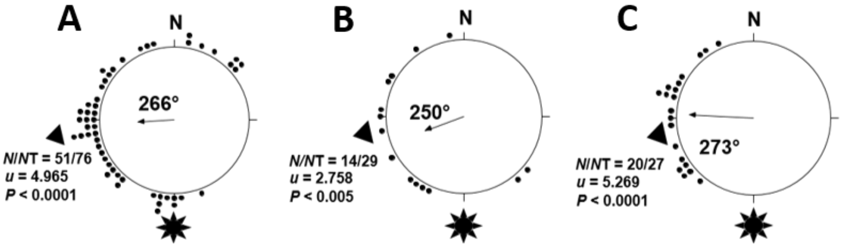

Fig. 10. Releases under the grey filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 4. for further information.

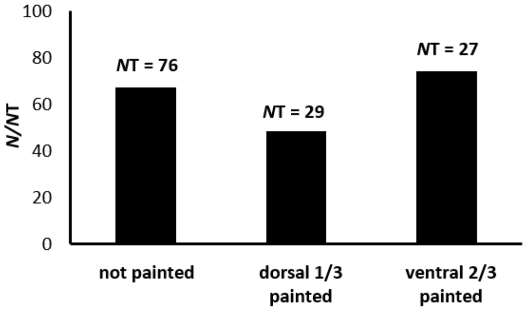

Fig. 11. Radially-oriented frequencies in releases carried out under the grey filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

Experiments conducted with the superimposition of the CR39 filter over the bowl and the sun visible revealed a mean orientation of not treated individuals in accordance with the seaward direction of their home beach (deflection of the mean vector = 23°) (Fig. 12A). Similarly, animals with either the 1/3 dorsal (Fig. 12B) or the 2/3 ventral (Fig. 12C) region of their eyes painted orientated correctly toward their escape direction; in both cases, the deflection of the mean vector was modest (8° and 13° respectively). Frequencies of radially-oriented individuals registered in different tests do not differ statistically each other (*G* = 1.086, df = 2, *P* = NS, G test) (Fig. 13).

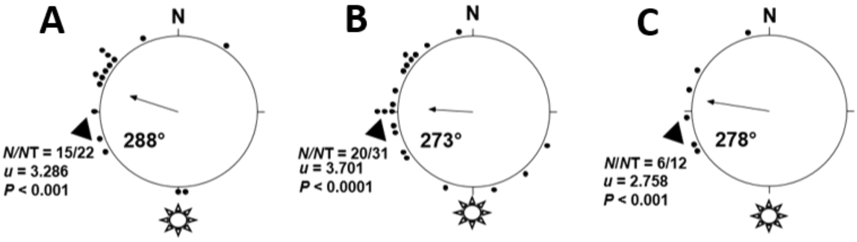

Fig. 12. Releases under the CR39 filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 4. for further information.

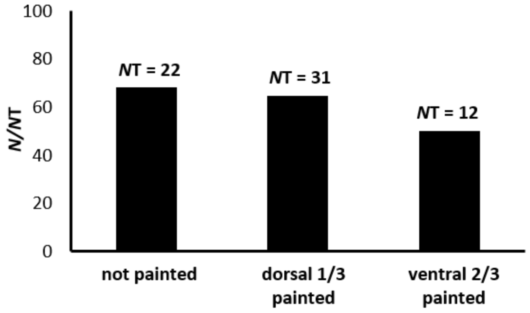

Fig. 13. Radially-oriented frequencies in releases carried out under the CR39 filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

In tests carried out under the CR39 filter and without the vision of the sun allowed untreated individuals (Fig. 14A) and sandhoppers with the 2/3 ventral region of their eyes painted (Fig. 14C) orientated significantly toward the expected direction (deflection of the mean vectors = 36° and 20° respectively); instead, animals with the 1/3 dorsal region of their eyes painted (Fig. 14B) were uniformly distributed (although 8/22 = 36% individuals tested directed correctly toward their escape direction) . Even in this case, frequencies of radially-oriented individuals occurring in trials carried out with different groups of sandhoppers do not differ statistically each other (*G* = 0.737, df = 2, *P* = NS, G test) (Fig. 15).

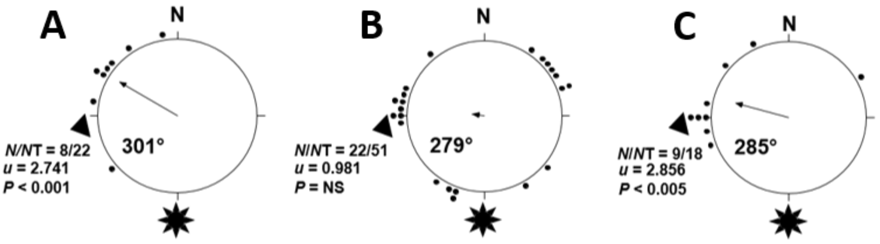

Fig. 14. Releases under the CR39 filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 6. for further information.

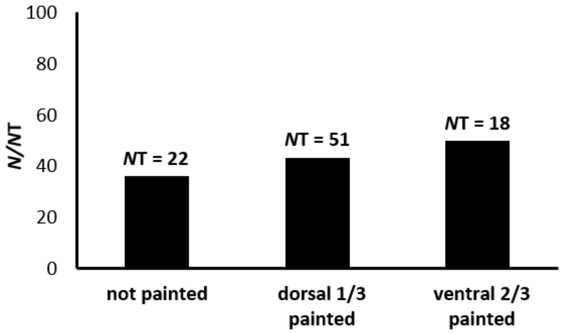

Fig. 15. Radially-oriented frequencies in releases carried out under the CR39 filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

In releases carried out under the blue filter and with the sun visible each group of sandhoppers tested exhibited a mean orientation in accordance with their escape direction. In any case, the deflection of the mean resultant vector was modest (not treated individuals = 25°, Fig. 16A; sandhoppers with the 1/3 dorsal region of their eyes painted = 28°, Fig 16B; sandhoppers with the 2/3 ventral region of their eyes painted = 24°, Fig. 16C). Comparisons between trials carried out with different groups of animals revealed statistically significant differences in the frequencies of radially-oriented individuals (*G* = 6.784, df = 2, $P < 0.05$, G test) (Fig. 17).

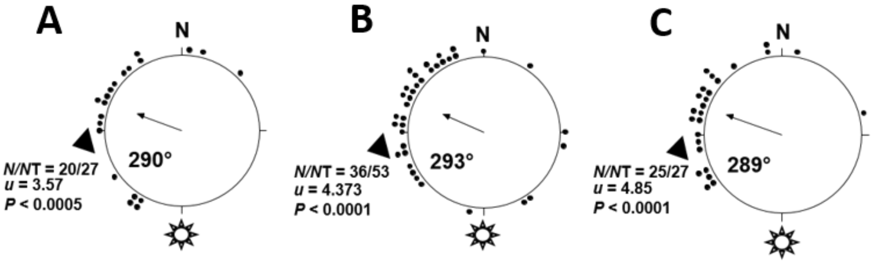

Fig. 16. Releases under the blue filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 4. for further information.

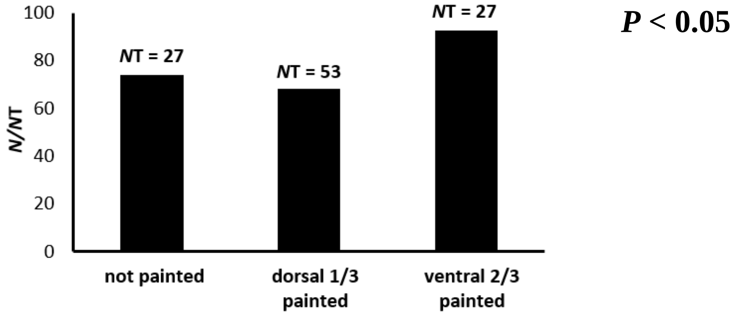

Fig. 17. Radially-oriented frequencies in releases carried out under the blue filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

Even when tested under the blue filters and the sun screened both untreated individuals (Fig. 18A) and sandhoppers with either the dorsal 1/3 (Fig. 18B) or the ventral 2/3 (Fig. 18C) region of their eyes painted orientated significantly toward the seaward direction of their home beach (deflection of the mean vector = 7°, 52° and 34° respectively). Also in this case frequencies of radially-oriented individuals recorded in different trials differ statistically each other ($G = 15.888$, df = 2, $P < 0.001$, G test) (Fig. 19).

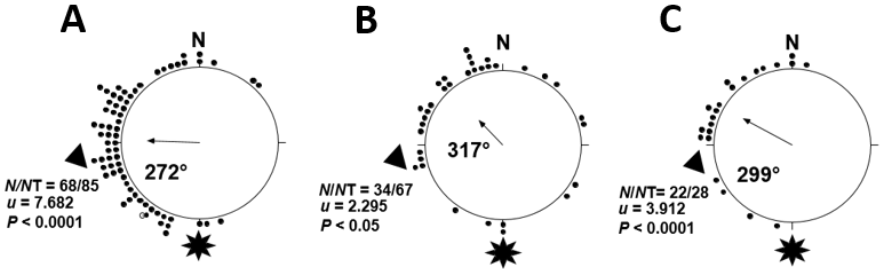

Fig. 18. Releases under the blue filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 6. for further information.

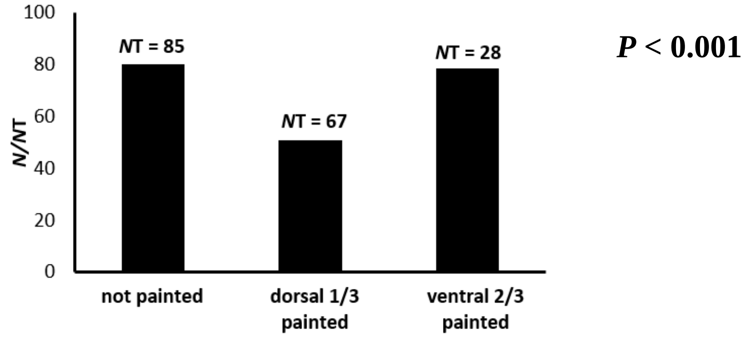

Fig. 19. Radially-oriented frequencies in releases carried out under the blue filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

In releases conducted under the green filter superimposed to the bowl and the vision of the sun allowed, both individuals with their eyes not painted (Fig. 20A) and those with the 1/3 dorsal region of their eyes painted (Fig. 20B) were randomly distributed. Instead, animals with the 2/3 ventral region of their eyes painted significantly orientated toward the expected direction (difference between the mean vector and the expected direction = 10°) (Fig. 20C). Any statistically significant difference in the frequencies of radially-oriented individuals between trials carried out with different groups of sandhoppers was recorded (*G* = 0.127, df = 2, *P* = NS, G test) (Fig. 21).

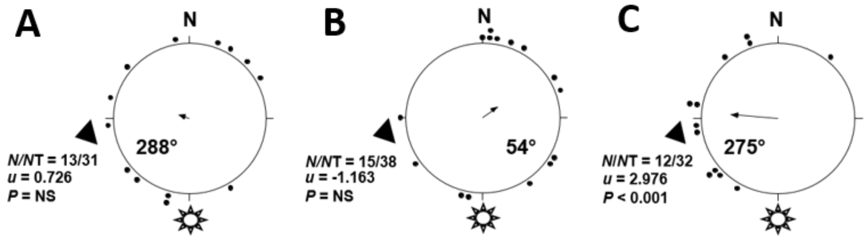

Fig. 20. Releases under the green filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 4. for further information.

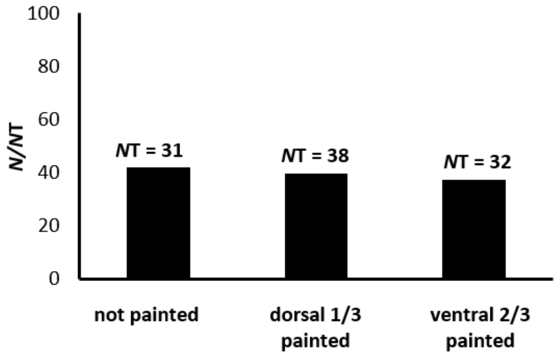

Fig. 21. Radially-oriented frequencies in releases carried out under the green filter with the sun visible. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

In tests carried out under the green filter and with the sun screened untreated animals (Fig. 22A) and individuals with the 1/3 dorsal region of the eyes painted (Fig. 22B) were uniformly distributed. Sandhoppers with the 2/3 ventral region obscured tested in the same conditions were mainly (14/15 = 93%) unable to perform directional choices (Fig. 22C). However, any significant difference in the frequencies of radially-oriented individuals was revealed between groups of sandhoppers tested (*G* = 3559, df = 2, *P* = NS, G test) (Fig. 23).

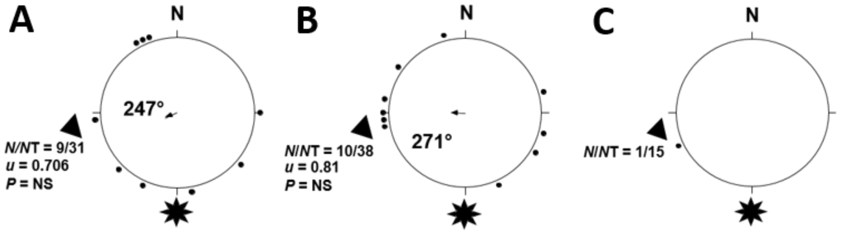

Fig. 22. Releases under the green filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted. See Fig. 8. for further information.

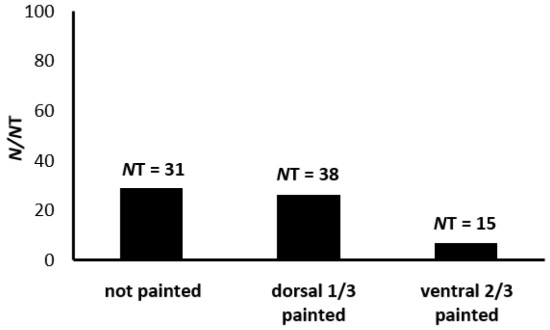

Fig. 23. Radially-oriented frequencies in releases carried out under the green filter with the sun screened. (A) not treated individuals, (B) individuals with the 1/3 dorsal region of their eyes painted and (C) individuals with the 2/3 ventral region of their eyes painted.

Conclusions

Results do not allow to clearly demonstrate a spatial distribution of the photoreceptors (sensitive to UV-blue and green wavelengths respectively) within the compound eye of *T. saltator*. In fact, releases of sandhoppers with different areas of their eyes obscured show that the experimental treatments do not compromise their celestial orientation when the perception of wavelengths shorter than 450 nm was not prevented.

However, tests conducted under the grey and blue filters, as well as those carried out in conditions of natural sky visible, show that animals with the dorsal margin of their eyes painted generally exhibited a greater difficulty to orientate with respect the other groups of sandhoppers released. Indeed, the frequencies of radially-oriented individuals recorded were in most cases lower. These results point out the importance of the upper portion of the eye in the perception of celestial orienting factors suggesting a regionalization of the visual capabilities in this species.

Since individuals released under the grey filter, transmitting only 23.5% of the total amount of light, maintained their capability to orientate toward the expected direction, we can exclude that the decrease in light transmitted by colored filters affects the celestial orientation of *T. saltator*.

Sandhoppers tested under the CR39 filter (blocking wavelengths shorter than 400 nm) mainly exhibited a mean orientation in accordance with the expected direction. However, in all trials conducted with the sun screened (also those carried out with individuals not subjected to experimental treatments), low frequencies ($\leq 50\%$) of radially-oriented individuals were recorded. Therefore, wavelengths shorter than 400 nm, although not essential, seem to play a role in the correct perception of celestial cues involved in the zonal recovery of this species.

As expected, sandhoppers tested under the green filter (selecting $450 \text{ nm} < \lambda < 590 \text{ nm}$ wavelengths) were unable to perform chronometrically-compensated orientation. Moreover, they exhibited a general difficulty in performing directional choices and in any case low frequencies (< 45%) of radially-oriented individuals were recorded. Only sandhoppers whose ventral 2/3 region of their eyes was obscured, although mainly non-radially oriented (20/32 = 63%), exhibited a mean orientation in agreement with their seaward direction in conditions of sun visible. This result is ambiguous since even not treated individuals totally lost their ability to orientate correctly and in contrast with results obtained in previous investigations (Ugolini et al., 1996).

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CHAPTER 4

OPTICAL AND FUNCTIONAL CHARACTERIZATION OF *TALITRUS SALTATOR* (CRUSTACEA, AMPHIPODA) COMPOUND EYE

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In preparation

Abstract

The supralittoral sandhopper *Talitrus saltator* is well known to rely on several celestial cues, such as the sun, the moon and the luminance pattern of the sky, to reach the damp belt of sandy beaches by following the shortest route (i.e. the sea-land axis of the shore). Although the importance of the vision in the orientation of this species, the optical and fuctional features of its compound eyes have been scarcely investigated so far. In general, it has been believed that sandhoppers possess straight ommatidia suitable to optimize light absorption efficiency. Here, we show that both crystalline cones and rhabdoms are in fact hook-shaped in almost all of regions of the eye (except for the dorsal area) of *T. saltator* while ommatidia exhibit a wide field of view. We speculate that reflecting pigment cells, identified around crystallne cones and retinular cells, facilitate the passage of the light through the optical axis and thus improve the photoreception efficiency. Moreover, the anatomical differences pointed out between dorsal ommatidia and those occurring in the rest of the eye support the existence of a Dorsal Rim Area (specialized in detectecting polarized light) in sandhoppers and constitute the framework for future investgations to fully elucidate this argument.

Introduction

The main ecological challenge that the supralittoral sandhopper *Talitrus saltator* (Montagu, 1808) must overcome is to return as quick as possible to the belt of damp sand of the beach, where it lives buried during the day, in order to avoid dehydration and/or submersion. In its zonal recovery, this species moves along the sea-land axis of the shore and relies on several celestial cues, such as the sun, the moon and the luminance pattern of the sky (Papi & Pardi, 1953, 1954, 1959; Pardi & Papi, 1953; Ugolini et al., 2009), by compensating their azimuthal variations during the 24 h period to maintain a constant effective direction.

Of course, the vision plays a fundamental role in the perception of the visual stimuli used as orienting factors by this species. To date, the spectral sensitivity of sandhoppers to UV-blue (λ = 390-450 nm) and green (λ = 500-550 nm) light (Ugolini et al., 2010) as well as their capability to detect polarized light in the blue wavelengths (λ = 435 nm) range (Ugolini et al., 2013) are well demonstrated through behavioral and electrophysiological studies. On the contrary, only little is known on the optical and functional features of the eye of this species. Morphologically (see Ugolini et al., 2010 for a review), the compound eye of *T. saltator* is formed by about 300 ommatidia (Gallien & David, 1936) each consisting of a dioptric region, composed of a hexagonal cornea (15 µm thick) and a crystalline cone (length = 60 µm, maximum diameter = 35 µm), and a photoreceptive region, constituted by a rhabdom. Rhabdoms (length = 70 µm in central ommatidia) are formed by the microvillar expansions (rhabdomeres) of five retinular cells (R1-R5); four of these cells (R1-R4) are large and arranged in two sets (R1-R3 and R2-R4) with their rhabdomeres orientated perpendicularly each other, whereas the fifth cell is shorter than the other ones. *T. saltator* possesses a fused-type rhabdom since its rhabdomeres are strictly joined each other (Ercolini, 1965).

In general, ommatidia were believed to exhibit straight optical axis in order to optimize their photoreception efficiency. Indeed, such an anatomical structure could facilitate the convey of the light into the optical system (Fig. 1). However, no investigation has been carried out to confirm this supposition.

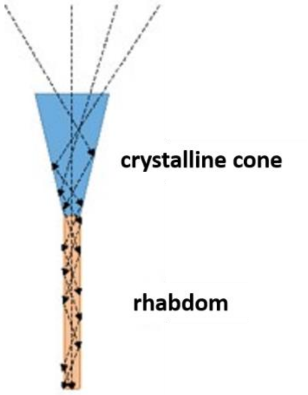

Fig. 1. Schematic representation of an ommatidium characterized by a straight optical axis.

Furthermore, the capability of this species to detect polarized light (although its actual role in the zonal recovery of sandhoppers is unknown) arises the argument on the existence within its eye of a dorsal rim area (DRA), similar to that occurring in other arthropods, exhibiting specific morphological adaptions suitable to enhance polarization sensitivity (see Labhart & Meyer, 1999 for a review).

Therefore, the aims of this work are 1) to investigate the optical and functional fetaures of the compound eye of *T. saltator* and 2) to evaluate eventual morphological differences between ommatidia present in the dorsal area and those occurring in the rest of the eye in order to facilitate the identification of a region specialized in the perception of polarized light within the eye of this species.

Material and methods

Morphology

The heads of several specimens of *T. saltator* were first removed from the bodies with a small piece of razor blade in a primary fixative solution (2.5% glutaraldehyde in 0.1 mol sodium cacodylate buffer, pH 7.4), placed in a refrigerator (4 °C) for 2 h and rinsed twice in cacodylate buffered solution. The eyes were gently isolated by using a small piece of razor blade and fine tweezers and post-fixed for 2 h with 1% OsO_4 in the same buffer solution. After being rinsed, the fixed eyes were dehydrated through a graded series of ethanol solutions, transferred to propylene oxide and embedded in Epon 812.

For light microscopy, serial semi-thin (1 μm thick) sections were cut with a Leica IM UC7i microtome (Leica Biosystem, Germany) and stained with 1% toluidine blue.

3D reconstructions of ommatidia were obtained by using photos taken from each section by using light microscope. Briefly, each image was aligned by Image J (https://imagej.nih.gov/ij/) and stored in Photoshop (http:// http://www.photoshop.com/) to define the opacity and color of each voxel. Voxels were then rendered and photos were volume-rendered by TEMography (System in Frontier Inc.).

For transmission electron microscopy, sections were cut with a Leica IM UC7i microtome (Leica Biosystem, Germany) and picked up with 100-mesh copper grids. They were double-stained with 1% uranyl acetate and 0.1% lead citrate solution for 20 min and 30 min, respectively. Observations were performed using a JEOL JEM-1400 transmission electron microscope (JEOL Inc., USA).

Electrophysiology

The antennae, legs and posterior half of the body of several living specimens were cut off and the anterior half of the body was introduced into a pipette tip. The head was fixed with 1-Tetradecanol first and firmly fixed with resin/paraffin or beeswax/resin mixtures. The posterior half of the body was filled with seawater to maintain the animals alive and a silver wire was introduced into the pipe for the reference electrode. *T. saltator* mounted as described above survived for a few hours. A small triangular hole, equivalent to 5-10 facets, was made in the dorsal part of the cornea using a fresh chip of a razor blade, in order to facilitate the electrode insertion.

Responses were measured with a pre-amplifier (Nihon Koden MEZ-8301) and a Nihon Koden VC-11 Memory oscilloscope with Nihon Koden AVH-11 highgain amplifier. Data were digitalized and recorded by a PC via a data logger (NR-500, Keyence Co.).

An Ushio power supply (XB-50101AA-A) was used to run a 500W Xenon arc lamp (Ushio Inc., Type UXL-500D-0). Quartz lenses produced a parallel beam of light that passed through a heat absorption filter, and one of a set of 16 narrowband interference colour filters (Vacuum Optics Corp., Japan IF-S) scanning a range of wavelengths from 330 nm to 660 nm. These monochromatic stimuli were adjusted by using a quartz neutral density filter (optical wedge) so that they contained an equal number of photons. At all wavelengths the maximum intensity available at the eye was 9.0 x $l0^{13}$ quantacm^{-2}/s^{-1}, a value which corresponds to about the brightness of a dull day. The quartz light guide led the stimulating light into a Faraday cage and to the preparation; stray light was prevented from exciting the eye by enclosing the light beam in felt. The light-emitting end of the light guide was attached to a perimeter device and held 5 cm from the eye of the specimen. A photodiode which was used to monitor these operations revealed no significant ripples.

A relation between intensity and response (V/logI curve) was first determined for single retinular cells by delivering a series of flashes (200 msec), with their intensity increasing in 0.2 log-unit steps. The field of view of retinular cells was then roughly determined by moving the source of light, delivering flashes of suitably adjusted constant intensity and duration, either horizontally or vertically through the optical axis of the retinular cells in 0.25° steps. The responses measured were converted into relative sensitivity values by normalizing them with respect the V/logI curve. Finally, the amplitude of the acceptance angle of light (considered as the maximum incidence angle of a ray hitting a retinular cell which allows the light to be guided into its optical axis) was defined by taking into account values of relative sensitivity greater than 50%.

Results

Fig. 2A shows the frontal view of *T. saltator*. Observations of dorso-ventral sections conducted by light microscope allow us to clearly detect the longitudinal axis of ommatidia (from the crystalline cone to the rhabdom) only in the dorsal margin of the compound eye, while elsewhere rhabdoms were hardly detectable. However, ommatidia seem to be hook-shaped in almost in all regions of the eye; only in the dorsal margin they exhibit a straighter shape (Fig. 2B).

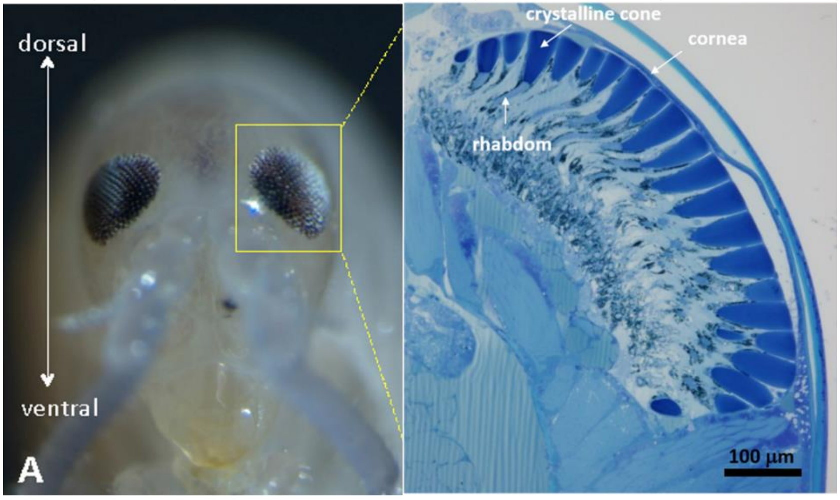

Fig. 2. Light microscope observations. Frontal view of *T. saltator* (A) and dorso-ventral semi-thin section of the left eye.

Observations of the dorsal margin of the eye conducted by using the transmission electron microscope pointed out that ommatidia occurring in this region exhibit straight crystalline cones and rhabdoms. Furthermore, even the five retinular cells contributing to the rhabdom have been detected (Fig. 3).

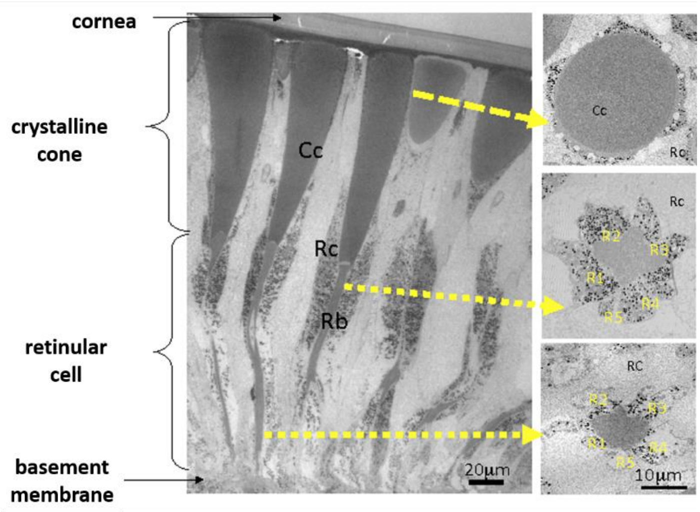

Fig. 3. Transmission electron microscope observations of the dorsal edge of the compound eye. Cc, crystalline cone, Rc, retinula cell, Rb, rhabdom.

3D reconstructions of the ommatidial structure confirmed main differences in shape between ommatidia occurring in the dorsal edge (straight, Fig. 4A) and those located elsewhere (bent, Fig.4B).

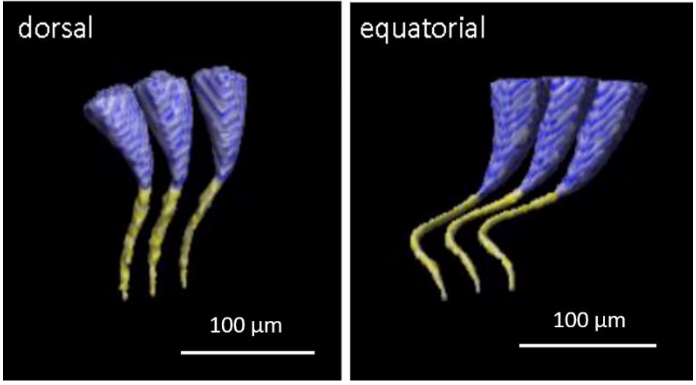

Fig. 4. 3D reconstruction of the structure of ommatidia occurring in the dorsal and the equatorial region of the compound eye of *T. saltator*.

To directly observe the shape of crystalline cones, the cornea was removed and they were separated from the rest of the compound eye by gently pipetting in the physiological saline. Crystalline cones showed several varieties in size and shapes; small to big and straight or hook-shaped forms. However, most of the crystalline cones isolated exhibited a curved shape (Fig. 5).

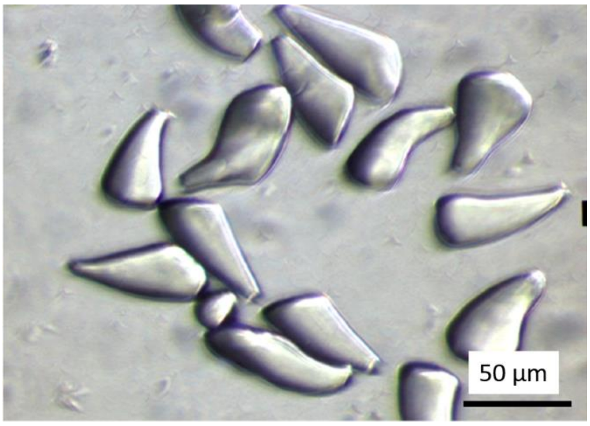

Fig. 5. Variability in shape and size of isolated crystalline cones.

Observations conducted by transmission electron microscope revealed that both crystalline cones and rhabdoms are surrounded by reflecting pigment cells (RpC) (Fig. 6A-B).

Reflecting pigment cells occurring between crystalline cones were also observed in whole eyes by light microscope (Fig. 6C) after the removal of the cornea. Their color appeared clear white and analyses of the refecting substances pointed out high reflectance from UV to near IR wavelengths (Fig. 6D).

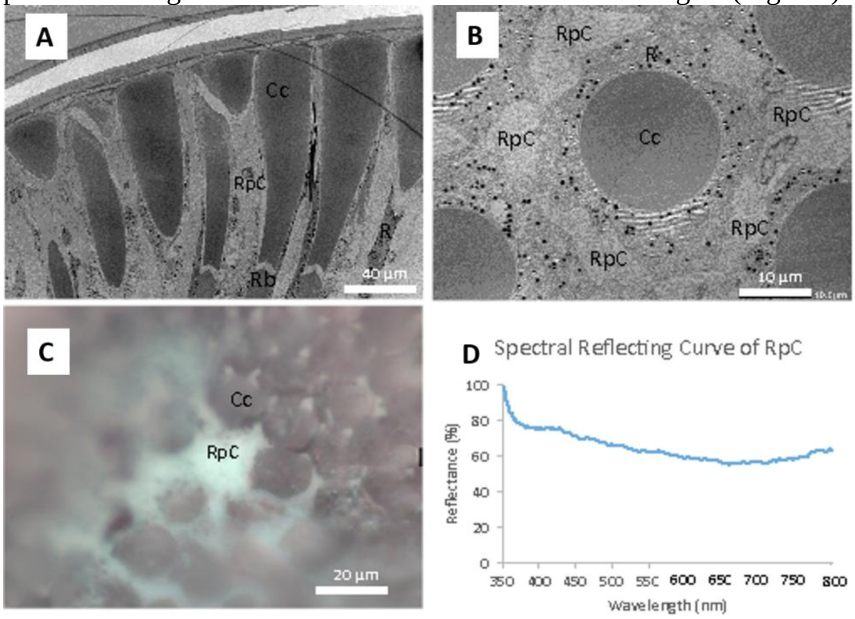

Fig. 6. Reflecting pigment cells surrounding crystalline cones and retinular cells. (A) Transmission electron microscope observations (longitudinal section), (B) transmission electron microscope observation (transverse section), (C) light microscope observation and (D) spectral reflecting curve of reflecting pigment cells. Cc, crystalline cone, RpC, reflecting pigment cell.

Finally, our electrophysiological recordings revealed a wide field of view of ommatidia occurring in the compound eye of sandhoppers. Indeed, the acceptance angle of light, measured both horizontally and vertically with respect the longitudinal axis of the retinular cells, equals approximately to 40 degrees (Fig. 7).

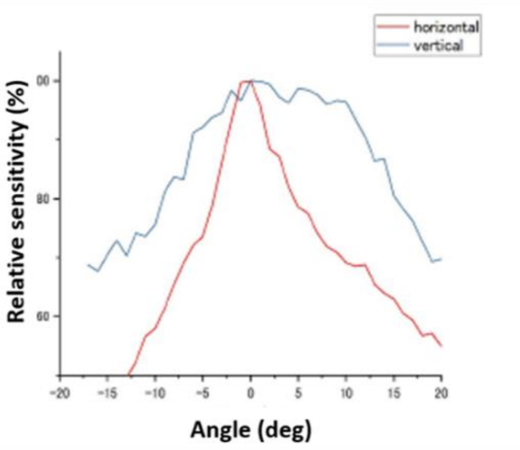

Fig. 7. Electrophysiological recordings: acceptance angle of light of *T. saltator* ommatidia.

Conclusions

This work deepens our knowledge on the morphological structure of the compound eye of *T. saltator* and constitutes a preliminary insight into the optic and functional features of the visual system of this species.

Our observations and 3D reconstructions reveal that crystalline cones and rhabdoms are typically hook-shaped in almost all regions of the eye (except for the dorsal area). This discovery is in contrast with the model proposed by Ercolini (1965) and apparently suggest a scarce photoreception efficiency of bent ommatidia since such an anatomical structure could highly reduce the amount of light conveyed into the optical system (Fig. 8A).

However, electrophysiological recordings showed that ommatidia exhibit a wide visual angle. These results, along with the identification of reflecting pigment cells (RpC) surrounding these anatomical structures, allow us to suppose that the RpC facilitate the light to pass through the optical axis widening the field of view of bent ommatidia (Fig. 8B) and thus enhancing the light absorption efficiency.

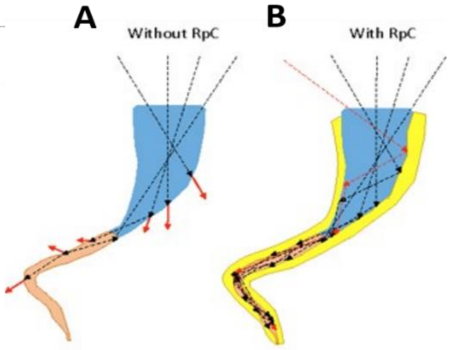

Fig. 8. Light path in bent ommatidia of the compound eye of *T. saltator*. (A) ommatidium without RpC and (B) ommatidium with RpC. RpC, reflecting pigment cells.

Finally, the differences revealed in the shape between ommatidia occurring in the dorsal edge and those present in the rest of the eye support the existence in this species of a dorsal rim area (DRA) morphologically specialized in the perception of polarized light. Indeed, straight optical axis could enhance the detection of the e-vectors pattern of the sky and straight ommatidia constitute a typical feature of the DRA of many arthropods (see Labhart & Meyer, 1999). Of course, further analyses are necessary to fully assess the actual involvement of these ommatidia in processing polarized light.

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CHAPTER 5

EVIDENCE FOR DISCRETE SOLAR AND LUNAR ORIENTATION IN THE BEACH AMPHIPOD *TALITRUS SALTATOR* MONTAGU (CRUSTACEA, AMPHIPODA)

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Abstract

Animals that use astronomical cues to orientate must make continuous adjustment to account for temporal changes in azimuth caused by Earth's rotation. For example, the Monarch butterfly possesses a time-compensated sun compass dependent upon a circadian clock in the antennae. The amphipod *Talitrus saltator* possesses both a sun compass and a moon compass. We reasoned that the time-compensated compass mechanism that enables solar orientation of *T. saltator* is located in the antennae, as is the case for Monarch butterflies. We examined activity rhythms and orientation of sandhoppers with antennae surgically removed, or unilaterally occluded with black paint. Removing or painting the antennae did not affect daily activity rhythms or competence to orientate using the sun. However, when tested at night these animals were unable to orientate correctly to the moon. We subsequently measured circadian gene expression in the antennae and brain of *T. saltator* and show the clock genes *period* and *cryptochrome 2* are rhythmically expressed in both tissues, reminiscent of other arthropods known to possess antennal clocks. Together, our behavioural and molecular data suggest that *T. saltator* has anatomically discrete lunar and solar orientation apparatus; a sun compass, likely located in the brain and a moon compass in the antennae.

Introduction

Many organisms use visible objects in their natural environment, including celestial bodies, as reference points to orientate appropriately. However, because of Earth's rotation, celestial objects appear to move across the sky over the course of a 24 h daily cycle and so orienting using these cues necessitates continuous adjustment to maintain a constant direction. A classic model of this, the Monarch butterfly *Danaus plexippus*, uses the sun as an orientation cue during its remarkable annual migrations but to maintain a constant flight direction, compensates for azimuthal variation of the sun by means of a circadian timing mechanism (Froy et al., 2003). Remarkably, in *D. plexippus*, in addition to the circadian clock found in the central brain, an extra-cerebral clock localised in the antennae is essential for appropriate time-compensated solar orientation; the removal of antennae, or painting them black unilaterally, disables correct flight orientation (Reppert, 2007; Merlin et al., 2009; Guerra et al., 2012). The consensus arthropod model of the molecular circadian clock of fruit flies is centred on transcriptional-translational feedback loops where the positive transcription factors CLOCK (CLK) and CYCLE (CYC) drive the transcription of negative elements *period* (*per*) and *timeless* (*tim*) the cognate proteins of which accumulate in the cytoplasm, form a heterodimer and translocate to the nucleus to suppress their own expression, a cycle that lasts about 24 h owing to regulatory post-translational events (for detailed reviews see Allada & Chung, 2010; Mazzotta & Costa, 2016). Molecular characterisation of the circadian clock in the Monarch brain revealed a novel negative transcriptional feedback loop with CLK and CYC driving rhythmic transcription of *period*, *timeless* and *cryptochrome 2* (*cry2*), the protein product of the latter feeding back as the main repressor of CLK:CYC (Zhu et al., 2008; Markert et al., 2016). Later work determined that similar feedback loops were central to the timing mechanism found in the antennae but entirely separate from the brain clock. Indeed, Monarch antennal clocks can be synchronised, or entrained, to light and dark (L:D) cycles even when physically separated from the head of the animal (Merlin et al., 2009). The sandhopper *Talitrus saltator* is one of the best-known biological models for studies on compass mechanisms in littoral arthropods (Pardi & Papi, 1952). Since the Fifties, it has been demonstrated that supralittoral sandhoppers artificially or naturally displaced from the band of damp sand where they live during the day use multiple cues in their recovery to their preferred shore zone, following the shortest route (i.e. the sea-land axis of sandy beaches). This is important to minimize the effects of biotic and abiotic stressors present in that environment, such as predation, variations in temperature, salinity and relative humidity (Ugolini, 2003). For the same reasons, sandhoppers evolved nocturnal habits. In nature, *T. saltator* emerges from its burrows at night and performs inland excursions to forage, before returning to the belt of damp sand at the water's edge before sunrise. This rhythm of locomotor activity is endogenous and synchronized to the natural 24 h diurnal cycle by the dawn (Williams, 1980). However, because sandhoppers can also be displaced from their refuges during the day by natural and artificial disturbances, they must be able to compensate for the azimuthal variation of the celestial bodies over the entire day/night cycle. Previous studies have shown that the endogenous, circadian clock mechanism, regulating the rhythmic locomotor activity exhibited by *T. saltator*, is also involved in sun compass orientation behaviour (Ugolini, 2003). Intriguingly, nocturnal orientation using lunar cues appears to rely on a separate time-compensatory mechanism (Ugolini et al., 1999, 2012a). Comparison of the anatomical organization of Monarch time-compensated navigation and the behavioural orientation of sandhoppers raises the question of whether sandhoppers evolved anatomically discrete, dual systems to enable them to orientate at night-time and daytime to locate their preferred foraging and resting sites. To address this question, we performed (a) surgical removal or (b) unilateral occlusion of antennae with black paint and assessed the solar and lunar orientation of animals under natural light regimes in a zeroed horizontal component of the natural magnetic field. This experimental approach enabled us to test whether solar and lunar orientation is mediated by mechanisms in the antennae and to establish whether these are anatomically separate from the timing mechanisms that control daily locomotory activity rhythm. Moreover, we evaluate if the directional choice of antennaless sandhoppers is really based on the sun or moon compass instead of other compass cues (such as the geomagnetic field).

Material and methods

Animals and husbandry

Adult individuals of *T. saltator* were collected from a sandy beach in the Natural Park of Migliarino, San Rossore, Massaciuccoli, Pisa, Italy (43°40′ 03″ N, 10°20′ 29″ E), sea-land direction = 265°-85°, in June-July 2015 and June 2016. Experimental orientation releases were performed no more than 21 days from collection and were made under a clear sky and with an unobstructed view of the sun or moon. In the laboratory, animals were kept in Plexiglas boxes containing damp sand at ambient temperature (25 °C) and with an artificial photoperiod (L:D = 12:12) in phase with the natural L:D cycle (i.e. with the same mid-point for the light phase). We opted to maintain experimental animals on an L:D = 12:12 regime (i.e. 15° per hour of sun azimuthal variation) to avoid any influence of the hours of light on the speed of time-compensation of the azimuthal variation of the sun (see Ugolini et al., 2002). Food (universal dried food for fish, SERA Vipan, Heisenberg, Germany) was available *ad libitum*.

Sun compass experiments

Individuals were divided into: (1) intact animals, (2) animals with both first and second antennae surgically removed (Fig. 1A), and (3) animals with right antennae painted with black enamel (Fig. 1B).

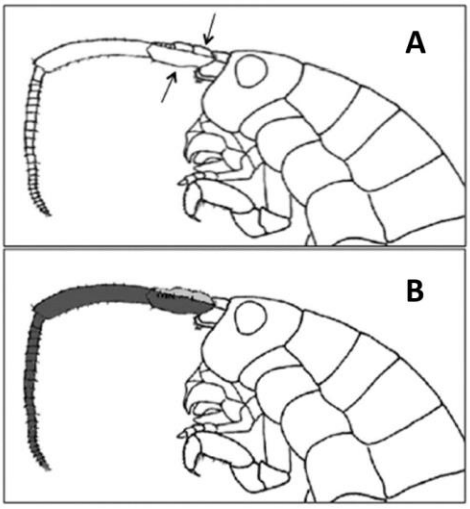

Fig. 1. Surgical removal of the antennae. (A) Schematic representation of the removal of the first and second antennae (arrows show the approximate position of cutting on first and second antennae), and (B) the unilateral painting of the first (light grey) and second (dark grey) antennae. Redrawn from Ruffo (1993).

Members of each group were relocated to the laboratory (with an L:D = 12:12 cycle in phase with the natural photoperiod) where activity was monitored using a micro-wave radar system (see Pasquali & Renzi, 2005 and Ugolini et al., 2012b for a detailed description of the device).

Experimental orientation releases were performed in Florence in June 2016, after at least 10 days of activity monitoring, between 1130 hours and 1300 hours. Taking into account similar experiments carried out on *D. plexippus* (Guerra et al., 2012), we also painted antennae with black enamel to test if discordant timing between antennae affects the sun or moon compass. Sandhoppers with right antennae painted with black enamel (Fig. 1B, Rainbow, Maimeri S.p.A., Mediglia, Milano, Italy) were tested 24 h after painting. It has previously been demonstrated that testing sandhoppers in small groups does not influence their directional choice (Scapini et al., 1981). Therefore, for each trial, 5-10 individuals were released into a transparent Plexiglas bowl, filled to a depth of about 0.5 cm with seawater. Seawater induces sandhoppers to rapidly orientate toward the landward direction of their home beach. The bowl was placed horizontally on a transparent plate so that the sandhoppers could be observed from below. The bowl and plate were mounted on a tripod and surrounded by a cylindrical screen 1-3 cm high allowing the animals in the bowl only the vision of the sun and sky (Ugolini & Macchi, 1988). In order to carry out releases with a zeroed (or very reduced) horizontal component of the natural magnetic field that could constitute a further orienting reference (Arendse & Kruyswijk, 1981), we equipped the tripod with a pair of Helmholtz coils (diameter = 64 cm, distance = 35 cm) supplied by a battery and regulated by an electronic rheostat. A single direction for each sandhopper was determined 2 minutes after the introduction of the animals to the bowl. Directions were measured from freeze-frame images recorded by a video camera placed below the bowl or read directly by the experimenter (error ± 2.5° in both cases).

Moon compass experiments

For moon compass experiments, animals were prepared exactly as for sun compass experiments; i.e. individuals were divided into: (1) intact animals, (2) operated without first and second antennae (Fig. 1A), (3) right antennae painted with black enamel (Fig. 1B). Tests were conducted in July 2015 in the same conditions as for sun compass tests, under full moon phase (95% illuminated fraction). The direction of each individual was recorded by an infra-red (IR) sensitive video camera placed below the bowl and measured from freeze-frame images, as previously described (Ugolini et al., 1999). The bowl was illuminated with an IR source, placed approximately 2 m from the bowl. Previous studies have shown that *T. saltator* is not sensitive to IR (Ugolini et al., 1996, 2010).

Clock gene expression analysis

Cerebral ganglia and antennae (first and second) were harvested separately from *T. saltator* after entrainment under 12:12 L:D cycles followed by a 24 h free-running period in DD. All tissues were preserved in RNAlater at −20 °C until extraction. Total RNA was extracted, reverse transcribed and subjected to qPCR using Taqman™ hydrolysis probes (Thermo Life Sciences, UK) and absolute quantitation according to previously published methods (Zhang et al., 2013).

Gene sequences for primer and probe design were obtained from the brain transcriptome of *T. saltator* (O'Grady et al., 2016). Data were expressed as copy number of each target gene transcript per copy number of the reference gene *arginine kinase*.

Statistical analysis

In all the orientation experiments only one direction per individual was recorded. Directional data were analysed using 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. To assess the non-uniformity of distributions the Rao's test has been used ($P < 0.05$ at least). Rhythmicity in clock gene expression was determined using Cosinor software (http://www.circadian.org/softwar.html) developed by Roberto Refinetti et al. (2007) and using default circadian parameters.

Results

Antennae are not required for sun-compass orientation

Initially, we reasoned that the time-compensated orientation mechanism of *T. saltator* resides in the antennae, as is the case for Monarch butterflies, and tested the behaviour of animals with antennae surgically removed or unilaterally painted black and under the sun to investigate whether they play a role in the solar orientation. Daily activity rhythms of all animals remained unaffected following antennal removal (Fig. 2A-B). Directional choices of intact individuals showed a clear bimodality in accordance with the Y-axis of their home beach (Fig. 2C) and the mean landward direction of these animals was very similar to those of sandhoppers without antennae (only 14° different, Fig. 2D). Moreover, releases of animals with unilateral first and second antennae obscured with black paint also showed a mean direction in close agreement with the landward direction of their home beach (30° different from expected, Fig. 2E).

Therefore, our results demonstrate that antennae are not essential in the solar orientation of *T. saltator* since their removal or unilateral painting does not disrupt the of sea-land direction finding based on the sun compass (Fig. 2C-E).

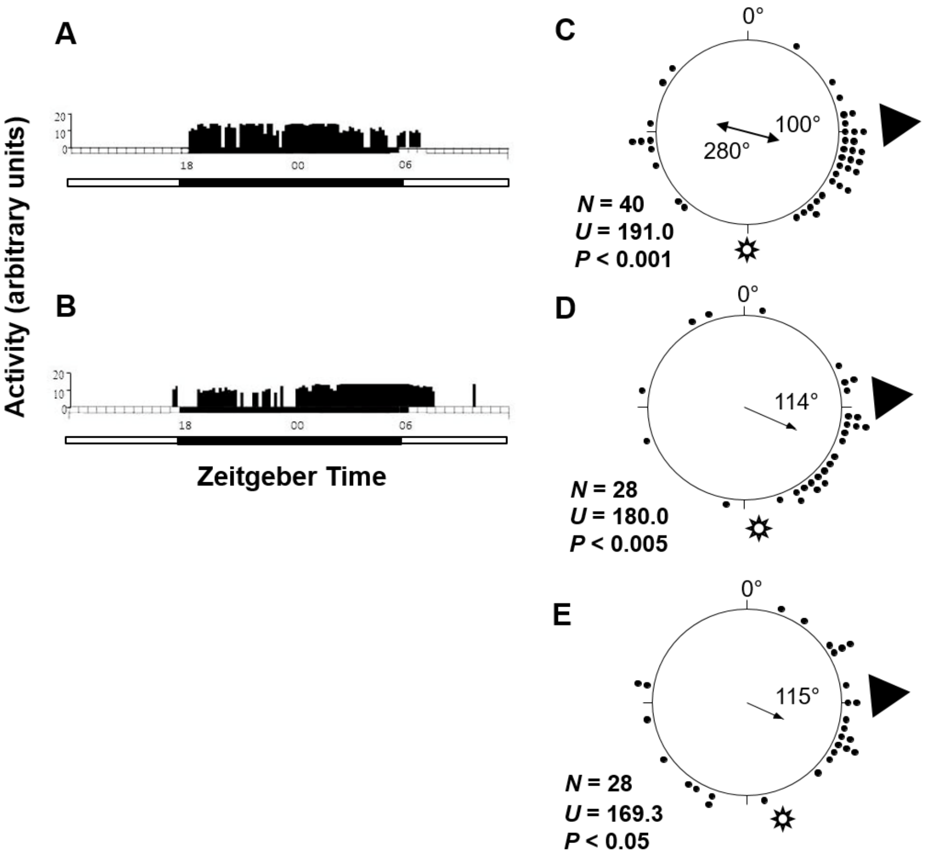

Fig. 2. Daily locomotor activity and solar orientation are unaffected by antennal ablation. Locomotor activity of (A) intact and (B) antennae ablated animals in L:D = 12:12. Black and white bars indicate light and dark respectively. The quantity of activity (recorded simultaneously from a group of twenty individuals in each experiment) is expressed in arbitrary units. Distributions obtained testing: (C) intact, (D) antennaless and (E) right antennae black painted animals. 0°, North (zeroed magnetic field); arrow inside the distributions: mean vector and angle (length of mean vectors: 0 to 1 = radius of the circle); black dots: sandhoppers' directions (each dot corresponds to the direction of one individual); black triangles outside the distributions: landward direction of sandhoppers' home beach. Sun symbol: solar azimuth at the time of the test. *N*, sample size. *U*, Rao's test values with probability levels (*P*).

Antennae are required for moon-compass orientation

Next, we tested lunar orientation of sandhoppers by performing the same experimental releases at night, under a full moon (95% illuminated fraction). Fig. 3A clearly shows that whilst the intact individuals exhibit a bimodal distribution in accordance with the expected orientation of animals migrating up or down-shore (i.e. the sea-land axis of their home beach), individuals lacking first and second antennae (Fig. 3B), or with their first and second antennae unilaterally painted black (Fig. 3C), did not orientate in the expected direction (59° and 122° different, respectively) but instead displayed a photopositive orientation directed toward the source of light (i.e. the moon). Thus, sandhoppers lacking antennae or unilaterally blinded with paint do not lose competence to orientate using the sun as a chronometrically compensated astronomic cue but are unable to use the moon as a reference in the same way.

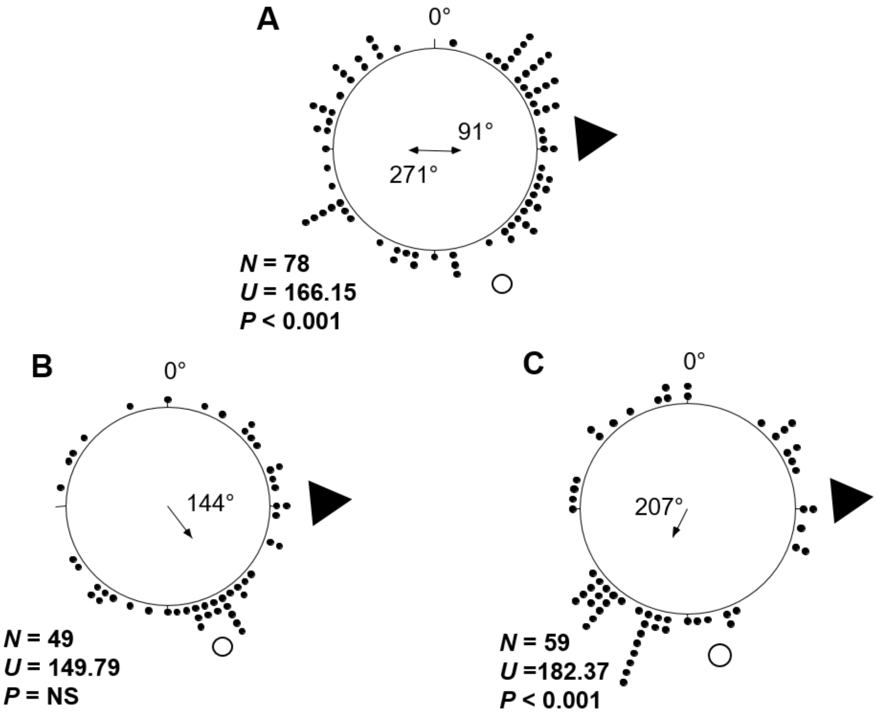

Fig. 3. Antennal manipulation disrupts lunar orientation. Distributions obtained testing: (A) intact, (B) antennaless and (C) right antennae painted black animals held under L:D = 12:12 (with lighting in phase with the natural photoperiod). See Fig. 2 for explanations.

Both the brain and antennae express canonical clock gene transcripts

The consensus model of the core circadian clock mechanism is based on transcriptional-translational autoregulatory feedback loops (Allada & Chung, 2010), the molecular elements of which are well conserved across diverse taxa (Young & Kay, 2001; Kyriacou et al., 2008). A feature of this system in many organisms is that core clock transcripts show rhythmic changes in abundance over a 24 h period. Therefore, demonstrating cycling expression of clock genes in the antennae of *T. saltator* in free-running (constant darkness, DD) conditions would support the notion that these appendages have the potential to keep time. We used Taqman™ qPCR assays to measure the temporal expression profiles of the canonical clock gene homologues *period*, *timeless*, *clock*, and *cryptochrome 2* in animals held in DD following entrainment in L:D = 12:12 (Fig. 4).

Cosinor analysis of gene expression profiles revealed rhythmicity in *Talper* (peak expression between CT15-21) and *Talcry2* (peak expression between CT3-15) in the brain. *Timeless* expression in the brain was not shown to be rhythmic by Cosinor analysis. *Talclk* was not significantly rhythmic. In the antennae, only *Talper and Talcry2* cycled (Cosinor), with mRNA abundance peaking at around CT15-18, similar to that of the brain. However, antennal *Talcry2* expression was in antiphase to that in the brain with peak abundance between CT21 and CT3 and low levels at CT9-CT15.

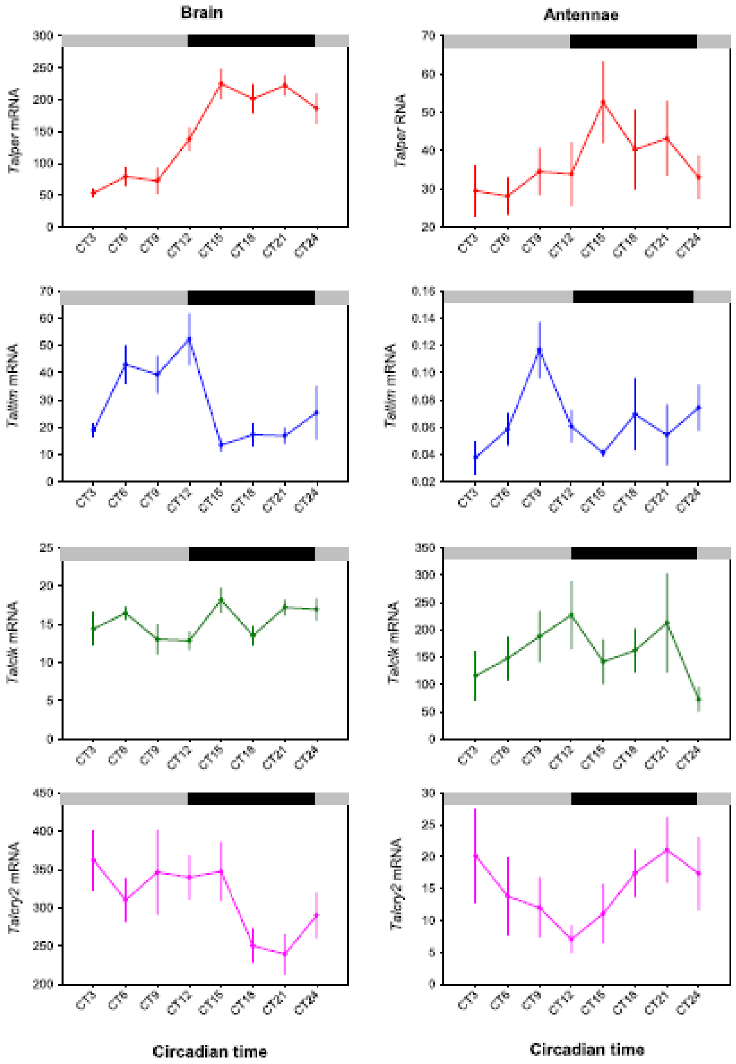

Fig. 4. Brain and antennae show rhythmic clock gene expression in constant darkness. Circadian clock gene transcripts were detected in the brain (left panel) and

antennae (right panel). Quantification of these transcripts over a 24 h period revealed rhythmic accumulation of *Talper* and *Talcry2* in both tissues by cosinor analysis: Brain, *Talper*, *F* = 17.42, *P* = 0.0067, *Talcry2*, *F* = 18.84, *P* = 0.0056; Antennae, *Talper*, *F* = 6.61, *P* = 0.04, *Talcry2*, *F* = 15.18, *P* = 0.009). *Talclk* and *Taltim* were not rhythmic within a circadian period. Data are expressed as copies RNA per copy of the reference gene *arginine kinase*. Data are mean +/− sem from 6 biological replicates of 5 pooled heads. Grey and black bars show time of expected light and dark respectively.

Discussion

We set out to test whether the solar and lunar orientation mechanisms in *Talitrus saltator* share a common anatomical apparatus located in the antennae, the site of the time-compensated solar mechanism in the Monarch butterfly, *Danaus plexippus*. We show that the ability of sandhoppers to orientate using the sun is not perturbed by removal of, or unilaterally obscuring the antennae but, night-time compass orientation under moon-light is compromised. We also show that the antennae and brain exhibit rhythmic expression of core circadian genes that suggest these animals may have an antennal clock involved in lunar orientation. The results obtained by Reppert and co-workers on the Monarch butterfly and our data presented here raise the question of whether, in arthropods, antennae are obligatory for time-compensated solar and/or lunar orientation. For the sun compass, it appears that this is not the case since some lycosid spiders possess a sun compass but lack antennae (Tongiorgi, 1969). Moreover, our tests show that *T. saltator* can orientate correctly using a sun compass after removal or unilateral occlusion of the antennae. These results are in contrast to *D. plexippus* which loses the ability to orientate effectively after these treatments. The case for lunar orientation is somewhat similar; among the very few species of arthropods in which time-compensated lunar orientation has been demonstrated (Pardi & Papi, 1952; Pardi, 1954; Papi, 1960; Tongiorgi, 1969) only the crustaceans *T. saltator* and *Tylos europaeus* possess antennae, whilst the spider *Arctosa variana* lack these appendages. In the ant *Formica rufa* (Jander, 1957) and the earwig *Labidura riparia* (Ugolini & Chiussi, 1996) the time compensation of the moon azimuth has not been definitively demonstrated, and while any general phylogenetic conclusions remain speculative, it appears that time-based moon orientation may be a feature of coastal crustaceans. The existence of lunar and solar orientation mechanisms in the same organism is not without precedence; in *T. saltator* this has been demonstrated using behavioural experiments (Ugolini et al., 1999). The existence of timing mechanisms synchronised with lunar phases and lunidian cycles was established many years ago (Naylor, 2010), but whether phenotypes occurring on monthly or circatidal (12.4 h) cycles were governed by dedicated non-circadian time-keepers remained unresolved. Recently however, evidence from behavioural, genetic and pharmacological experiments in the crustacean *Eurydice pulchra* and polychaete worm, *Platynereis dumerilii* has emerged supporting the notion that circatidal and circalunar phenotypes are orchestrated by timekeepers separate from the circadian clock (Zantke et al., 2013; Zhang et al., 2013). Given the well-documented role of clock genes, including *per* and *cry2* in transcriptional-translational regulatory feedback loops in other species, their function in Monarch butterfly time-compensation (Merlin et al., 2009) and temporal gating of olfactory function in insect antennae (Merlin et al., 2006, 2007; Schuckel et al., 2007), we postulate that *T. saltator* antennae also have a timing mechanism. In flies, light-entrained antennal clocks that govern olfactory sensitivity operate independent of the central oscillator and visual system (Plautz et al., 1997; Tanoue et al., 2004) and in Monarch butterflies 'cryptochrome-centric' clocks in the central brain and antennae play different roles in circadian timing and navigation (Merlin et al., 2009; Zhu et al., 2013). Our demonstration of daily oscillations in clock gene expression in the antennae of *T. saltator* reveals the existence of a putative molecular timekeeper in these tissues. Notwithstanding mounting evidence for the importance of post-transcriptional and post-translational events in the molecular clock (Lakin-Thomas, 2006; Merrow et al., 2006; Mehra et al., 2009), and indeed rhythms in the absence of transcription (O'Neill, 2011), it is a feature of many oscillatory systems that rhythmicity in clock gene expression reflects the functioning of the clock mechanism; in crustaceans free-running rhythms in core cock genes have been reported as evidence of a clock mechanism (Zhang et al., 2013; Sbragaglia et al., 2015). In the absence of available reagents and the apparent difficulty of measuring clock proteins in small crustaceans *in vivo* (Zhang et al., 2013), the clear daily fluctuations in *period* and *cryptochrome 2* are an indication that the antennae have the competence for time-keeping. This pattern of *Talcry2* expression in the antenna is reminiscent of antennal *cry2* in butterflies (Merlin et al., 2009) and although speculative at this stage, the antiphasic expression of *Talcry2* in the antennae and brain presents the tantalizing prospect that, the phase differences in these discrete tissues reflect solar versus lunar timing function. At present, we can not rule out the possibility that the moon compass in *T. saltator* is not exclusive to the antennae. Nevertheless, our experiments show that whilst the antennae of *T. saltator* are not necessary for solar orientation, they are important in the functioning of a moon compass. Moreover, we demonstrate rhythmic expression of canonical clock gene transcripts in the brain and antennae, illuminating the possibility that the latter might have an important function in time-keeping, separate from that of daily locomotor rhythms. Taken together, our data suggest that the antennae accommodate a time-keeping component of the lunar compass, discrete from the solar clock, which is probably located in the brain or eye. Future work will aim to elucidate the cellular localization of these putative clocks in *T. saltator.*

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CHAPTER 6

INSIGHTS ON THE INTERACTIONS AMONG ANTENNAL OSCILLATORS IN *TALITRUS SALTATOR* (CRUSTACEA, AMPHIPODA)

Introduction

To return as quick as possible to the belt of damp sand in which it remains buried during the day the supralittoral sandhopper *Talitrus saltator* (Montagu, 1808) orientates along the sea-land axis of the beach, perpendicular to the shoreline. This species in order to avoid dehydration that can quickly lead animals to death exhibits nocturnal habits. Environmental disturbing factors can cause even diurnal activity and sandhoppers direct seaward when exposed to high temperature and low humidity conditions whereas submersion induces landward orientation. In its zonal recovery, *T. saltator* is known to rely on both the sun and the moon (Papi & Pardi, 1953, 1954, 1959; Pardi & Papi, 1953; Ugolini 1999, 2007). Of course, to use celestial cues implies endogenous mechanisms accountable for the chronometric compensation of their azimuthal variations throughout the 24 h period. Separate time-keepers governing the sun and the moon compass systems have been revealed in this species (Ugolini et al., 1999).

In the Monarch butterfly *Danaus plexippus*, the sun compass mechanism is regulated by antennal time-keepers independent of the central oscillator and directly entrainment by the external photoperiod (Merlin et al., 2009). However, it seems that timing information from each antenna are downstream integrated and conflicting inputs compromise the functioning of the chronometric mechanism (Guerra et al., 2012). In fact, whereas the unilateral removal of antennae does not affect the capability of individuals to orientate to the sun, their unilaterally black-painting (preventing light entrainment) disrupts the solar orientation of this species.

Recent studies conducted on *T. saltator* (Ugolini et al., 2016) showed a discrete anatomical localization of the time-keepers underlying the two compass mechanisms and suggested the existence even in this species of antennal oscillators involved in time-compensated celestial orientation. Indeed, behavioural tests demonstrated that the surgical removal of antennae highly affects the capability of sandhoppers to orientate under the moon (whereas their solar orientation was not affected by this treatment). Biomolecular analyses revealed also that these appendages have the competence for time-keeping by pointing out rhythmic expression of canonical clock gene homologues. However, these investigations did not clarify whether only either first or second antennae exhibit pace-makers involved in the functioning of the moon compass mechanism exhibited by *T. saltator*.

Moreover, it has been shown that sandhoppers with right antennae black-painted could not orientate to the moon effectively and exhibited positive phototactic responses (Ugolini et al., 2016). These results suggest that even in this species inputs from right and left antennae undergo a downstream integration. However, it has not been shown whether individuals with antennae of one side of the head removed can still orientate under the moon since results obtained could be due to factors other than the conflicting entrainment of antennal oscillators. Indeed, researchers did not evaluate the behaviour of sandhoppers with left black-painted appendages. Since in some Hymenoptera it has been revealed the lateralization of visual and olfactory functions (Letzkus et al., 2006, 2008; Rogers & Vallortigara, 2008; Anfora et al., 2011), we can not be excluded that this species exhibits time-keepers only in their right antennae.

Therefore, the aims of this works are: 1) to assess whether the functioning of the moon compass mechanism in *T. saltator* involves selectively either first or second antennae and 2) to evaluate if sandhoppers lacking right antennae are still able to orientate correctly to the moon.

Material and methods

Adult individuals of *T. saltator* were collected on two sandy beaches located near Torre Burano (South Tuscany, Grosseto, Italy, 42°24’04’’N, 11°22’44’’E; sea-land direction = 193°-13°) and in the Natural Park of Migliarino, San Rossore, Massaciuccoli (Pisa, Italy, 43°40’03’’N, 10°20’29’’E; sea-land axis = 265°-85°) in June 2016 and 2017 respectively. Behavioural tests were carried out within a week from collection.

Sandhoppers were maintained in the laboratory in Plexiglas boxes containing damp sand at room temperature (25°C) and under an L:D cycle = 12:12 corresponding to the natural photoperiod (sunrise = 0600 hours, sunset = 1800 hours). Universal dry food for fish (SERA Vipan, Heisenberg, Germany) were constantly available.

Releases were conducted in a confined environment using an apparatus similar to that described by Pardi and Papi (1953) and lately modified (Ugolini & Macchi, 1988) (Fig. 1). It consisted on a tripod supporting a transparent Plexiglas plate (diameter = 30 cm) with a bowl (diameter = 20 cm) placed on into which individuals were released. A goniometer was set under the bowl to measure directions assumed by sandhoppers. The vision of the surrounding landscape was prevented using a circular Plexiglas screen (diameter = 30 cm, height = 6 cm). Directional choices performed by individuals were detected from freeze-framed images obtained using an infra-red sensitive video-camera placed under the device.

Fig. 1. Experimental device used in behavioural tests.

Groups of 10-12 animals were released at a time into the bowl since it has been shown that they do not influence each other in performing directional choices (Scapini et al., 1981). 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.

Releases of sandhoppers with either first or second antennae removed

Individuals collected on the beach near Torre Burano were subjected to the selective surgical amputation of either first or second antennae and were tested at least 24 h after the treatment.

Tests of solar and lunar orientation were conducted under the natural sky and sun/moon. To avoid any interference of the geomagnetic field, its horizontal component was zeroed by equipping the tripod with a couple of Helmholtz coils (diameter = 64 cm, distance = 35 cm) regulated by an electronic rheostat.

Diurnal experiments were conducted in Florence (43°46’45"N, 11°14’46"E) between 0830 and 0900 hours (sun azimuthal variations = 98°-100°).

Instead, nocturnal experiments were carried out 20 km away from Florence (to avoid the city albedo), under a full moon phase (0.99 illuminated fraction) between 2230 and 2300 hours (moon azimuthal variations = 136°-145°). Since it has been demonstrated that *T. saltator* can not perceive IR wavelengths (Ugolini et al., 1996, 2010), an electric torch with an IR filter (λ = 830 nm) was set 2 m from the device to provide adequate illumination.

In both tests of solar and lunar orientation, the bowl containing individuals was filled to a depth of 0.5 cm with seawater to rapidly induce sandhoppers to orientate landward (13°).

Releases of sandhoppers with right antennae removed.

Individuals collected on the shore in the Natural Park of San Rossore were subjected to the surgical removal of first and second right antennae and tested at least 24 h after the treatment.

Behavioural tests of solar and lunar orientation were conducted in the laboratory in conditions of artificial illumination (see Ugolini et al., 1998, 2005). The bowl containing sandhoppers were covered with an opaline Plexiglas dome (diameter = 80 cm) whose internal surface (the artificial sky) was illuminated using a fiber-optic illuminator (Schott KL1500). The end of the fiber bundle (diameter = 8 mm) was housed in a tube at the center of the dome so that the source of light was as close as possible to the center of the dome. The astronomical bodies were simulating by inserting the fiber bundle of a second fibre-optic illuminator in a circular hole (diameter = 4.5 mm) situated 45 degrees above the horizon on the surface of the dome.

Diurnal experiments were conducted between 1130 and 1200 hours (sun azimuthal variations = 152°-170°). To reproduce a scenario suitable to be recognized as a diurnal sky and thus induce sandhoppers to exhibit a proper solar orientation with respect the artificial sun (see Ugolini et al., 1998) the intensities of the artificial sky and sun were set at 172.125 μW/cm^2 and 68 μW/cm^2 respectively.

Nocturnal experiments were carried out in coincidence with the full moon phase (0.99 illuminated fraction) between 2130 and 2330 hours (moon azimuthal variations = 145-175°). The intensities of the artificial sky and moon were 13.56 μW/cm^2 and 40.125 μW/cm^2 respectively.

Tests of both solar and lunar orientation were conducted in dry conditions and individuals were dehydrated for a few minutes to induce them to quickly orientate seaward (265°).

Statistical analysis

Data were analysed according to the methods proposed by Batschelet (1981) for circular distributions. The length of the mean resultant vector and the mean angle was calculated. The Rao's test, U, was used to assess whether the distributions differed statistically from uniformity ($P < 0.05$ at least). The uni- or bimodality of the distributions was evaluated by multiplying the data by an index that varied from 1 (unimodal) to 4 (tetramodal). If the length of the mean resultant vector increased when multiplied by an index > 1, the distribution was considered non-unimodal and automatically opened at the points of greatest separation between animals' directional choices. The mean angles of the group were calculated separately (see Ugolini, 2001).

Results

Releases of sandhoppers with either first or second antennae removed

In tests of solar orientation intact individuals exhibited a mean orientation in good agreement with the landward direction of their home beach (deflection of the mean vector = 1°) (Fig. 2A). Similarly, sandhoppers lacking either first or second antennae mostly orientated toward their escape direction and in both cases the deflection of the mean vector was modest (4° and 7° respectively). However, in releases of individuals subjected to experimental treatments slight positive phototactic tendencies were observed (Fig. 2B-C).

When released under the moon the most part of intact sandhoppers (40/59 = 68%) orientated correctly toward the landward direction of their home beach (difference between the expected direction and the mean resultant vector = 11°). Instead, the rest of individuals (19/59 = 32%) exhibited phototactic responses toward the moon (Fig. 2D). On the contrary, animals subjected to the selective removal of first and second antennae did not perform directional choices consistent with a correct time-compensated lunar orientation and performed positive phototactic responses (Fig. 2E-F).

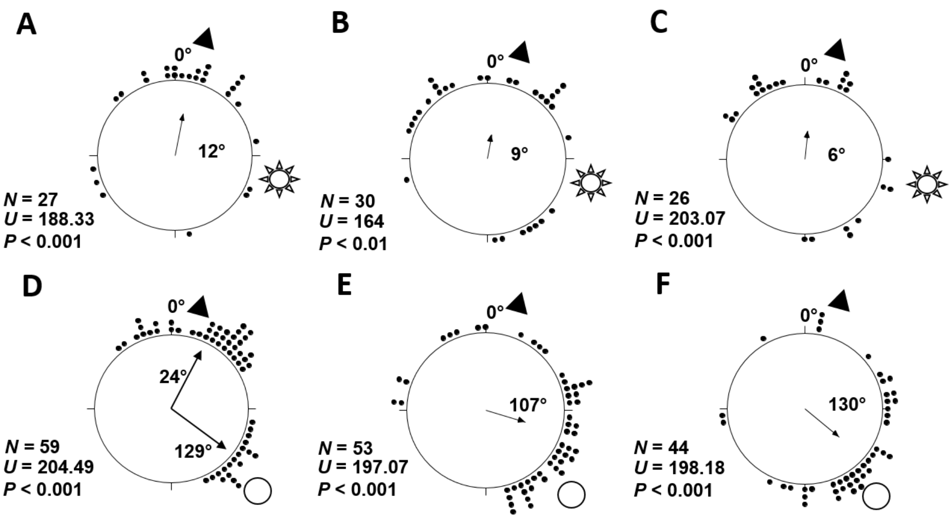

Fig. 2. Releases of sandhoppers under the natural sun (A, B, C) and moon (D, E, F). (A, D) intact individuals, (B, E) individuals with first antennae removed and (C, F) individuals with second antennae removed. 0°, North (zeroed geomagnetic field), black arrows, mean vector and angle (the length of the mean vector ranges from 0 to 1 = radius of the circle), black dots, sandhoppers' directions (each dot corresponds to the direction of one individual); black triangles outside the distributions, landward direction of animals' home beach. *N* number of radially oriented sandhoppers. The values of the Rao test, *U*, with their probability level, *P*, are also given.

Releases of sandhoppers with right antennae removed.

In tests of solar orientation conducted in conditions of artificial illumination intact individuals showed a mean orientation in good agreement with the seaward direction of their home beach (deflection of the mean resultant vector = 2°) (Fig. 3A). Similarly, sandhoppers subjected to the amputation of their right antennae directed properly toward their escape direction (difference between the mean vector and the seaward direction of their home beach = 31°) (Fig. 3B).

In nocturnal releases, intact individuals orientated effectively toward the expected direction with a modest deflection of the mean resultant vector (1°) (Fig. 3C). Even sandhoppers subjected to the removal of right antennae exhibited correct time-compensated lunar orientation and the mean vector was deflected with respect their (seaward) escape direction of only 11° (Fig. 3D). However, in both cases slight (non-significant) bimodal tendencies were observed since small groups of individuals directed toward the landward direction of their home beach.

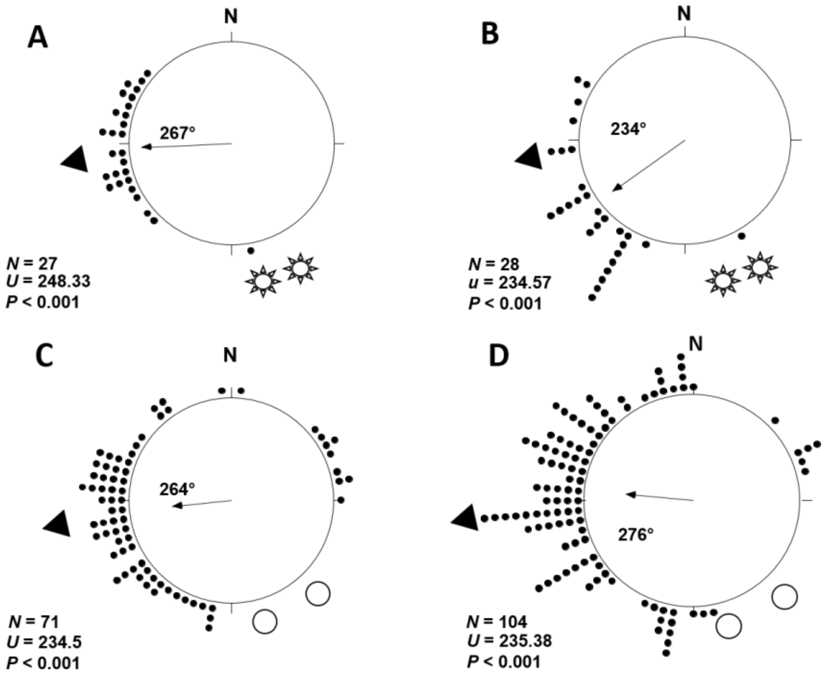

Fig. 3. Tests of solar (A, B) and lunar (C, D) orientation conducted in artificial conditions of illumination. (A, C) intact individuals and (B, D) individuals with right antennae removed. N, North; black triangles outside the distributions, seaward direction of animals' home beach. See Fig. 2 for further information.

Conclusions

This work reveals that time-keepers accountable for the correct functioning of the moon compass mechanism of *T. saltator* occur in both first and second antennae and oscillators located in either pair of appendages are both essential for the correct time-compensated lunar orientation of this species. In fact, individuals with either first or second antennae selectively amputated were unable to correctly orientate to the moon. On the contrary, when tested under the sun, sandhoppers subjected to the same experimental treatments performed directional choices in accordance with their escape direction, confirming also the discrete anatomical localization of that the oscillators regulating the two compass systems. Positive phototactic responses exhibited by small groups of individuals observed in these trials, as well as in releases of intact animals under the moon, do not constitute a new finding and can be attributed to the fact that tests were carried out in wet conditions. In fact, similar tendencies were revealed even in previous investigations (Ugolini et al., 1999, 2012) conducted following the same experimental procedures.

Releases of sandhoppers with right antennae amputated carried out in conditions of artificial illumination (under the Plexiglas dome simulating the sky) showed that the unilateral removal of these appendages does compromise neither solar nor lunar orientation. In fact, in both cases, individuals performed directional choices directed toward their escape direction (as well as intact animals). These results demonstrate that the loss of capability to orientate to the moon exhibited by animals with right antennae black-painted (Ugolini et al., 2016) was due not to a lack of timing inputs, partially clarifying also the argument on an eventual lateralization of antennal time-keepers. Therefore, it seems that also in *T. saltator* timing information from oscillators present in antennae of each side of the head are subjected to a downstream integration to generate a compass signal.

These results taken together show that inputs from oscillators occurring in first and second antennae are both necessary for the functioning of the moon compass system, but time-keepers located in antennae of one side of the head provide sufficient timing information for its regulation. Further investigations are required to assess the exact mechanism underlying the integration of inputs from each antenna.

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CHAPTER 7

LOCALISATION OF CORE CLOCK ELEMENTS IN *TALITRUS SALTATOR* (CRUSTACEA, AMPHIPODA)

Results reported in this section have been obtained in collaboration with Prof. David C. Wilcockson (Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, UK)

In the sandhopper *Talitrus saltator* (Montagu, 1808), biological clocks have been studied mainly from a behavioural point of view (see Pardi & Ercolini, 1986; Ugolini et al., 1999, 2007, 2016). Only recently (Ugolini et al., 2016), molecular analyses have been conducted to assess the circadian expression pattern of core clock gene homologues. However, the molecular and cellular basis of endogenous time-keepers in this species is unknown.

In this section, I report results obtained in the attempt to localize the cells within the protocerebrum of *T. saltator* expressing the canonical clock gene homologues *per* and *phd* (the latter the crustacean *pdf* homologue).

In this work, whole-mount immunological techniques (Tatutz & Pfeifle, 1989) were used that, contrary to other approaches based on antigen-antibody reactions, do not provide for the embedding of the samples thus avoiding eventual antigenic modifications.

Brains of adult specimens of *T. saltator* were dissected, fixed in Zamboni-Stefanini's solution (1967) and stored overnight at 4°C.

They were then washed 5X15' in PTX (phosphate buffered saline with 0.05-0.1% Triton X-100) to carefully remove the fixative and divided into two groups.

Each group was incubated overnight at 4°C with a different polyclonal antiserum (diluted 1:1000) containing primary antibodies raised against the genes products PER and PDH respectively (Davids Biotechnologie, GMBH).

Brains were then subjected to a second washing cycle in PTX and incubated overnight at 4°C with secondary antibodies (diluted 1:500 in PTX) labelled with fluorochromes. The antibody used to detect PHD was conjugated with fluorescein isothiocyanate (FITC), emitting in green wavelengths (495-519 nm), whereas that raised against antiPER primary antibodies was conjugated with an Alexa Fluor Dye (Thermo Fisher Scientific) emitting in red wavelengths (628 nm). To preserve their fluorescence samples were protected from light.

After a final washing cycle in PTX, brains were mounted onto microscope slides. A few drops of Vectashield® mounting medium (Vector laboratories, Burlingame, USA) were applied to the samples to prevent secondary antibodies from losing their fluorescence.

Immunoreactivity was identified using a confocal microscopy (Leica Microsystem GmbH).

Bilateral PER-positive cells, characterized by intense cytoplasmic staining, were localized in the medial region of the dorsal protocerebrum (Fig. 1).

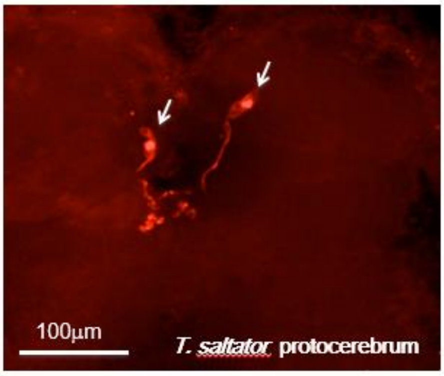

Fig. 1. PER staining in *T. saltator* protocerebrum. The white arrows indicated the soma of PER-positive cells.

PDH staining has been revealed in both the brain and the optic ganglia (Fig. 2). Soma of PDH-positive cells are located in the anterior protocerebrum and extensive arborization emanates from T-shaped axons to the optic lamina.

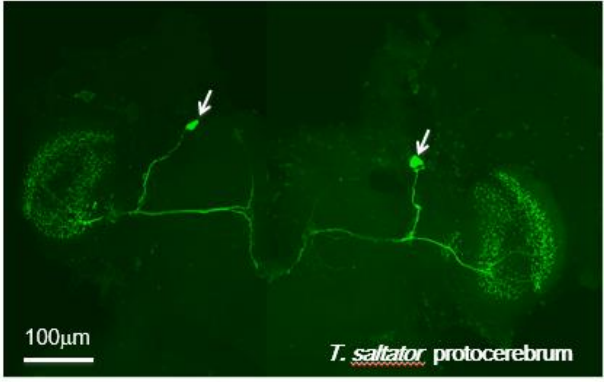

Fig. 2. PDH staining in *T. saltator* protocerebrum. The white arrows indicated the soma of PDH-positive cells.

The characterization of *per*- and *pdh*- expressing neurons in *T. saltator* is partially in accordance with results obtained in other species of arthropods. Indeed, comparative studies conducted in insects (see Helfrich-Förster, 2005) revealed in most of the species examined an almost exclusive cytoplasmatic staining of PER-positive cells. Only in *D. melanogaster*, it was observed alternate nuclear and cytoplasmatic labelling at different times over the 24 h period. However, in these species, per-expressing neurons have been localized in both the dorsal (dorsal neurons) and the lateral (lateral neurons) protocerebrum. Solely in the blow fly *Phormia regina* and the sphinx moth *Manduca sexta*, PER staining was limited to the dorsal region of the brain (Wise et al., 2002; Za´ vodska et al., 2003). Even in insects, PDF-positive cells exhibit an extensive arborization of their fibres connecting both the optic lobes and the protocerebrum. This arrangement is particularly suitable to transfer synchronizing information among different areas of the brain. Anyway, except for *M. sexta* (Homberg et al., 1991), these neurons are situated in the lateral protocerebrum and in *D. melanogaster* the same cells coexpress *per.*

In crustaceans, recent investigations conducted on *Eurydice pulcra* (Zhang et al., 2013) revealed *per*-expressing neurons characterized by a weak nuclear and an intense cytoplasmatic staining in both the dorsolateral and lateral protocerebrum.

These results provide a first description of the localization of clock elements on *T. saltator*. However, similar investigations could point out also connections among discrete anatomical districts implied in the functioning of biological clocks. In particular, recently (Ugolini et al., 2016), evidence for antennal time-keepers driving the moon compass system in this species was provided. These investigations suggested that timing information from oscillators occurring in appendages located on opposite sides of the head undergo a downstream integration to generate an output compass signal. However, the functioning of the integration mechanism is not known and immunological approaches could facilitate the localization of nervous connections between antennae and the central nervous system.

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CHAPTER 8

LIGHT SENSITIVITY IN THE ANTENNAE OF *TALITRUS SALTATOR* (CRUSTACEA, AMPHIPODA)

Results reported in this section have been obtained in collaboration with Prof. Takahiko Hariyama (Department of Biology, Hamamatsu University School of Medicine, Japan)

Recent studies conducted on the sandhopper *Talitrus saltator* (Montagu, 1808) suggested the existence of antennal oscillators regulating the chronometric mechanism of moon compass occurring in this species (Ugolini et al., 2016). In fact, besides behavioural investigations showing that antennae are essential to time-compensated lunar orientation, molecular analyses revealed substantial circadian oscillations in the expression of core clock gene homologues (*per* and *cry2*) in these appendages indicating their competence for time-keeping.

Further investigations suggested also that inputs from time-keepers occurring in antennae located in opposite sides of the head are downstream integrated and the discordant entrainment of oscillators disrupts the time-compensated lunar orientation of sandhoppers.

In this section, I report the results obtained in investigations addressed to assess the presence within the antennae of *T. saltator* of receptors sensitive to light, possibly involved in the synchronization of the time-keepers to the external cycle.

Electrophysiological recordings were conducted in order to point out the eventual electrical responsiveness of these appendages to light stimulation. To evaluate the origin of the responses, first and second antennae of adult individuals were cut from the scapus at different positions.

Samples were placed one at a time in a Faraday cage where the tip of the antennae was positioned in contact with filter paper moistened with seawater.

Extracellular recordings were conducted by inserting glass or tungsten electrodes into the antennal cavity; the thin silver wire protruding from the reference electrode was placed under the filter paper and covered with red wax to protect it from light.

Samples were irradiated with white light (duration of each stimulus = 400 msec; intensity = 10^5 μW/cm^2 corresponding to the illumination occurring during a bright day) by using a Xenon arc lamp (Model UXL-500D-O; Ushio Inc., Japan) connected to a power supply (Model XP-50101AA-A, Ushio Inc., Japan). Quartz lenses were used to produce a parallel beam of light that, after being passed through a heat absorption filter, was conveyed into the Faraday cage by a quartz light guide. The light-emitting end of the light guide was positioned 5 cm from the samples.

Electrical responses were measured by using a pre-amplifier (Nihon Kohden MEZ-8301) and a memory oscilloscope (Model VC-11 Nikon Kohden, Japan) connected to a high-gain amplifier (AVH-11 Nikon Kohden, Japan). Data were registered by a computer.

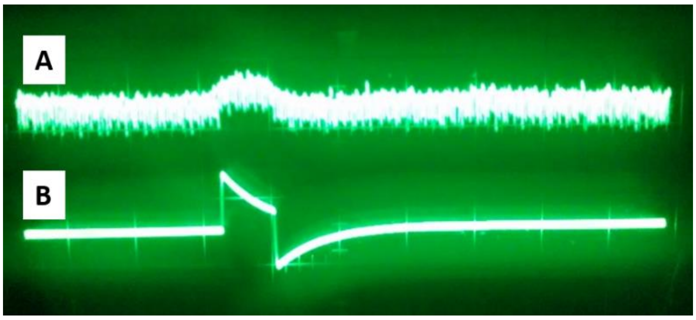

Fig. 1. Example of electrophysiological response to the light of *T. saltator* antennae. (A) light stimulation and (B) electrical response of whole second antenna.

Electrophysiological recordings clearly show electrical responses of the antennae to the light stimulus provided (Fig. 1). Similar results were obtained for both first and second antennae and independently of the cutting position.

This work suggests the existence of receptors sensitive to light in the antennae of *T. saltator*, likely widespread all along the appendages. However, further investigations are necessary to identify the nature of these photoreceptors and assess their actual involvement in the entrainment of antennal oscillators to the external cycle.

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Ugolini, A., Hölters, L.S., Ciofini, A., Pasquali, V. & Wilcockson, D.C. 2016. Evidence for discrete solar and lunar orientation mechanisms in the beach amphipod, *Talitrus saltator* Montagu (Crustacea, Amphipoda). *Scientific Reports*, **6**, 35575.

CHAPTER 9

SUN AN MOON COMPASSES IN *TYLOS EUROPAEUS* (CRUSTACEA, ISOPODA)

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Abstract

Experiments conducted in the Fifties provided first evidence that both the sun and the moon constitute astronomical compass cues in the zonal recovery of the supralittoral isopod *Tylos europaeus*. Whereas the sun-based orientation of this species has been thoroughly confirmed, no other investigation on its ability to orientate according to the moon has been carried out. In our studies, we tested adult individuals in a confined environment and demonstrated that: (1) animals can rely on the moon as a compass orienting factor despite the difficulties met in the time-compensated use of this celestial body; (2) lunar orientation is largely independent of the geomagnetic field; and (3) the moon compass seems to operate throughout the 24 h period thus even in the absence of the direct vision of the moon. Moreover, our preliminary tests conducted to assess the existence of discrete time-keepers regulating the sun and the moon compasses pointed out a partial separation between the two mechanisms even though further investigations are required to clarify this aspect.

Introduction

The main ecological challenge that the littoral isopod *Tylos europaeus* (Arcangeli, 1938) faces is to return to the belt of damp sand of sandy beaches where it lives buried during the day following the shortest route (i.e. the sea-land axis of the shore parallel to the coastline and thus characteristic for each population). This species exhibits nocturnal habits: at night animals emerge on the sand from their refuges and perform feeding excursions along the sea-land axis. However, environmental factors, like changes in the sea level or other biotic or abiotic stressors, can also cause diurnal activity (Giordani-Soika, 1954; Pardi, 1954, 1955; Matsakis, 1956; Mead, 1968; Tongiorgi, 1968): to escape from death animals must orientate seaward when exposed to dry conditions while landward orientation is induced by submersion. In some species of *Tylos*, dealing with the same ecological problem, it has been demonstrated that some local orienting factors, such as the slope of the beach and the vision of the landscape, are involved in their proper zonal recovery (Hamner et al., 1968; Mead & Mead, 1973).

To maintain a constant effective direction several species of arthropods are known to rely also on astronomical cues whose use implies endogenous chronometric mechanisms to properly compensate for their azimuthal variation throughout the 24 h period. For instance, the sandhopper *Talitrus saltator* can use both the sun and the moon to orientate along the sea-land axis of the shore (Papi & Pardi, 1953; Pardi & Ercolini, 1986; Ugolini et al., 1999, 2002, 2007; Ugolini, 2003) and it has been shown that separate time-keepers are involved in the functioning of the two compass systems (Ugolini et al., 1999, 2016).

Since the Fifties (Pardi, 1954, 1955) there has been evidence that even *T. europaeus* can rely on these two astronomical factors in its zonal recovery (Pardi, 1954). The solar orientation has been more recently confirmed in this species (Ugolini et al., 1995, 1997), as well as in conditions of artificial illumination

(Ugolini & Castellini, 2004). Moreover, the sun-compass mechanism seems to be innate since inexpert (born in captivity) individuals are able to orientate according to the same escape direction of the population they belong to (Ugolini et al., 1995). Instead, no further investigations have been conducted to assess its competence to rely on the moon as a compass cue. Time-compensated lunar orientation determines additional problems that animals have to overcome compared to the sun compass. In fact, during the lunar month the moon is not always visible (new moon), it never rises at the same hours and it does not always have the same shape.

Therefore, the aim of this paper is to evaluate the ability of this species to use the moon as a chronometric orienting factor in its zonal recovery and to assess the eventual relationship between the moon and the sun-compass systems.

Material and methods

We used adult individuals of *T. europaeus* belonging to two different populations collected on sandy beaches at Albegna (Southern Tuscany, 42°30′00′′N, 11°11′00′′E; sea-land axis = 268°-88°) and at Aleria (Corsica, France, 42°06′08′′N, 9°30′56′′E; sea-land axis = 271°-91°) over spring-summer 1998-2000. After their capture animals from Albegna were transported to the laboratory and maintained in plastic boxes containing wet sand under a light-dark (L:D) cycle correspondent to the natural photoperiod and at ambient temperature (25 °C). Food (dried fish food) was available *ad libitum*. Tests were conducted within a month from the animals' collection; nocturnal releases were carried out about 20 km away from Florence (Valigondoli 43°39′25″N, 11°11′ 09″E) to avoid the city's albedo.

Individuals from Aleria were tested immediately after their capture 100 m inland from their home beach. Experiments were carried out under the first quarter moon phase (48-50% illuminated fraction) and full moon phase (88-99% illuminated fraction) between 2100 hours and 2300 hours. Releases were performed in a confined environment. The device used (Ugolini & Macchi, 1988) consisted of a tripod supporting a horizontal transparent plate with a plexiglass bowl (diameter = 20 cm) placed on it. A goniometer was set under the bowl to detect animals' directional choices. Groups of 10 individuals were released into the bowl, filled to a depth of about 0.5 cm with artificial water (salinity = 33%) to induce them to orientate rapidly toward the landward direction of their home beach. Animals were tested in wet conditions because of the difficulties generally met in motivating them to orientate seaward during nocturnal experiments (due to the high humidity).

A couple of Helmholtz coils (diameter = 65 cm, distance = 35 cm) were used to manipulate the horizontal component of the magnetic field at will (e.g. Ugolini & Pardi, 1992). Tests were conducted under the natural sky. A cylindrical white screen (height = 6 cm) was used to prevent the vision of the surrounding landscape. Individuals' directional choices (error ± 2.5°) were measured from freeze-frame images recorded by a video camera placed under the bowl. An electric torch with an infrared filter (λ = 830 nm) placed 2 m from the bowl provided adequate illumination: this allowed the avoidance of eventual interferences from zenithal illumination or photographic flashes. A single direction for each individual was recorded after 2 min from their introduction into the bowl.

Deflection of the lunar azimuth

Animals belonging to the Aleria population were tested with the vision of the natural moon (full moon phase, azimuth from 150° to 179°) and with the natural moon screened out. In the latter case, to simulate the celestial body an electric torch, provided with a diffuser to reduce its brightness, was placed 2-3 m away from the bowl and projected from a fixed azimuth (270°; light intensity = 0.8 $\mu W/cm^2$). Tests were conducted both in the natural magnetic field and with the horizontal component of the magnetic field zeroed.

Manipulation of the horizontal component of the natural magnetic field

Animals from Albegna were tested under the natural moon (first quarter moon phase) in the natural magnetic field and with its horizontal component zeroed or deflected to the East. In these trials, the lunar azimuth ranged between 238° and 269°.

Animals' entrainment to clock-shifted solar and lunar cycles

We followed the same procedure adopted in similar experiments conducted on *T. saltator* (see Ugolini et al., 1999). Individuals were collected at Albegna during the new moon phase and divided into three groups respectively subjected for 14 days to: (1) an L:D cycle corresponding to the natural photoperiod and a false moon (an electric torch placed 40 cm from the boxes containing the animals, light intensity = 0.1 $\mu W/cm^2$; source diameter = 4 mm) in phase with the natural one; (2) an L:D cycle anticipated by 6 hours in respect of the natural photoperiod (new expected direction = 358°) and a false moon in phase with the natural one; and (3) an L:D cycle corresponding to the natural photoperiod and a false moon whose rise-set was anticipated by 7 days in respect of the natural one (new expected direction = 358°). Each group was tested in experiments of both solar and lunar (full moon) orientation. All trials were conducted with the horizontal component of the magnetic field zeroed.

Statistical analysis

Data were analysed according to the methods proposed by Batschelet (1981) for circular distribution. In any case, the length of the mean resultant vector and the mean angle were calculated.

To establish whether the distributions differed statistically from uniformity the Rao's test was used ($P < 0.05$ at least). The uni- or bimodality of the distributions was evaluated multiplying the data by an index that varied from 1 (unimodal) to 4 (tetramodal). If the length of the mean resultant vector increased when multiplied by an index > 1, the distribution was considered non-unimodal and automatically opened at the points of greatest separation between animals' directional choices. The mean angles of the group were calculated separately (see Ugolini 2001; Ugolini & Ciofini 2016).

Results

Deflection of the lunar azimuth

In tests conducted in the natural magnetic field under the natural moon (Fig. 1A) animals exhibited a mean orientation in good agreement with the expected landward direction of their home beach (the difference between the mean resultant vector and the expected direction was only 2°). Instead, when tested under the natural moon screened out and with the electric torch set at 270° (Fig. 1B) individuals orientated in accordance with the use of the fake moon as a compass cue. In fact, they assumed an angle to the light source correspondent to that expected to the natural moon and the mean resultant vector was close (20°) to the new expected direction (31°). Similar results were obtained in tests conducted with the horizontal component of the magnetic field zeroed. Indeed, in experiments carried out with the vision of the natural moon (Fig. 1C) animals properly orientated toward the landward direction of their home beach (the mean vector was deflected by only 11°). Instead, in tests conducted with the false moon set at 270° (Fig. 1D) individuals performed directional choices in agreement with the direction expected on the basis of a chronometrically compensated use of the fake celestial body (difference between the mean vector and the new expected direction = 5°).

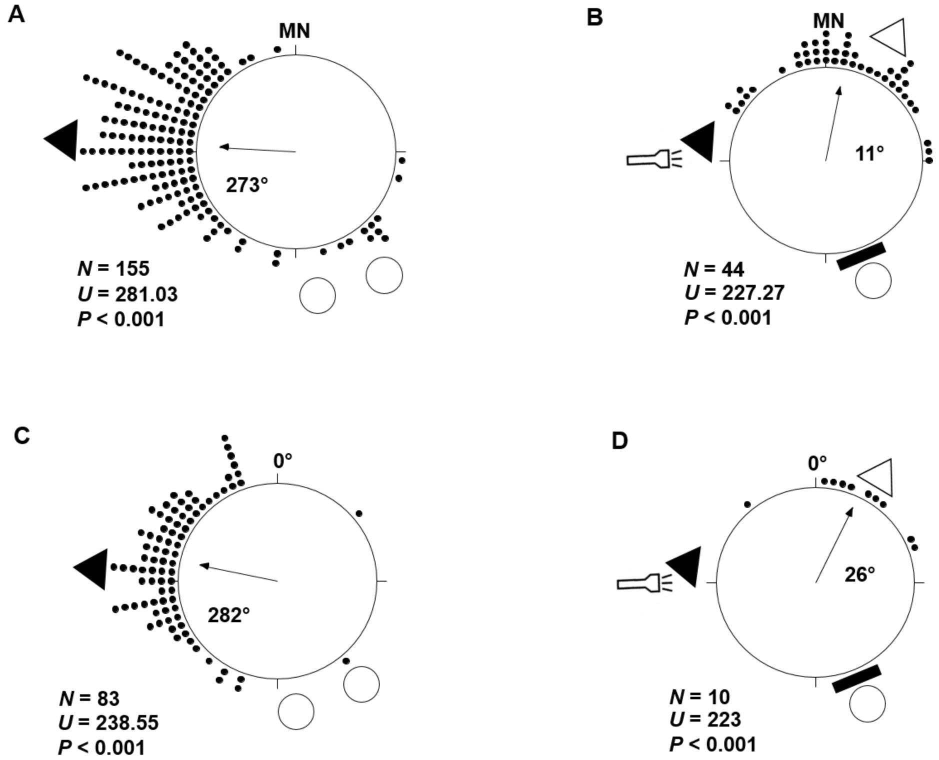

Fig. 1. Deflection of the lunar azimuth (Aleria population, full moon phase). Tests conducted in the natural magnetic field under the natural moon (A) and with the moon screened out and replaced with an electric torch at 270° (B); tests conducted with the magnetic field zeroed (North = 0°) under the natural moon (C) and with the moon screened out and replaced with an electric torch at 270° (D). MN, Magnetic North, black arrows, mean vector and angle (the length of the mean vector ranges from 0 to 1 = radius of the circle), black dots, animals' directions (each dot corresponds to the direction of one individual); black triangles outside the distributions, landward direction of animals' home beach, white triangles, new expected direction on the basis of the artificial moon azimuth. The symbols of the moon and of the torch indicate the lunar and the electric torch's azimuths at the time of releases respectively. *N*, number of individuals tested. The values of the Rao's test, U, with the probability level, *P*, are also given.

Manipulation of the horizontal component of the magnetic field

In tests conducted under the natural moon and natural magnetic field (Fig. 2A), animals exhibited directional choices in good agreement with the expected direction. Indeed, the mean vector is deflected only 15° from the landward direction of animals' home beach.

Similarly, individuals tested with both the horizontal component of the magnetic field zeroed (Fig. 2B) and deflected to the East (Fig. 2C) were correctly orientated toward the landward direction of their home beach. The difference between the mean vectors and the expected direction is 7° and 13° respectively.

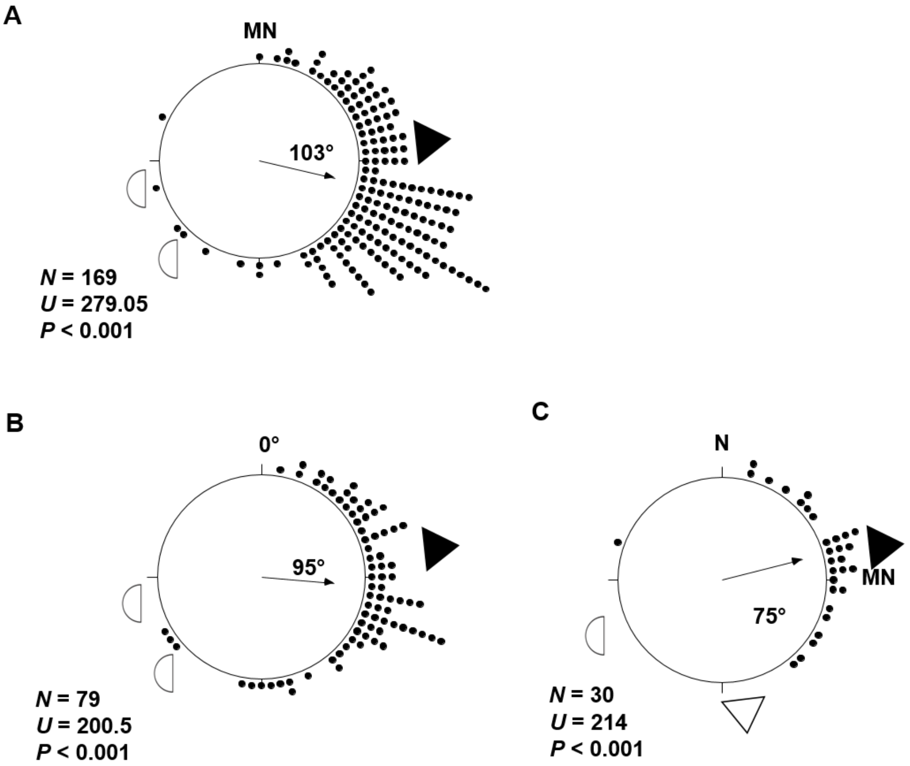

Fig. 2. Manipulation of the geomagnetic field (Albegna population, first quarter moon phase). Tests conducted with the vision of the natural moon and in the natural magnetic field (A), the magnetic field zeroed (B) and the Magnetic North deflected to the East (C). N, north, white triangle outside the distribution, expected direction according to the use of the magnetic field. See Fig. 1 for further explanations.

Animals' entrainment to clock-shifted solar and lunar cycles

Individuals previously kept under an L:D cycle corresponding to the natural photoperiod and a false moon in phase with the natural one orientated toward the landward direction of their home beach in tests of both solar (Fig. 3A) and lunar (Fig. 3B) orientation. The deflection of the mean resultant vector from the expected direction is modest in either case (19° and 14° respectively). Animals entrained to an L:D cycle anticipated by 6 hours in respect of the natural photoperiod and a false moon in phase with the natural one were significantly orientated toward the direction expected on the basis of the clock-shifting (= 358°) when tested under the sun (Fig. 3C) (difference between mean resultant vector and the expected direction = 8°). Instead, in tests of lunar orientation (Fig. 3D) the isopods were properly directed toward the landward direction of their home beach (deflection of the mean resultant vector = 10°). Individuals subjected to an L:D cycle in phase with the natural photoperiod and a false moon whose rise-set was anticipated by 7 days in respect of the lunar month performed a bimodal tendency in tests of solar orientation (Fig. 3E). Indeed, about half of the individuals tested (*N* = 16, 52%) were directed toward the landward direction of their home beach (difference between the mean vector and the expected direction = 1°) while the rest (*N* = 15, 48%) orientated toward the direction expected on the basis of the lunar phase-shifting (deflection of the mean vector = 3°). Moreover, when tested under the moon (Fig. 3F), they exhibited a significant orientation toward the expected direction according to the phase-shifting (358°), even though the direction of the mean vector differs from the new expected direction by 76°.

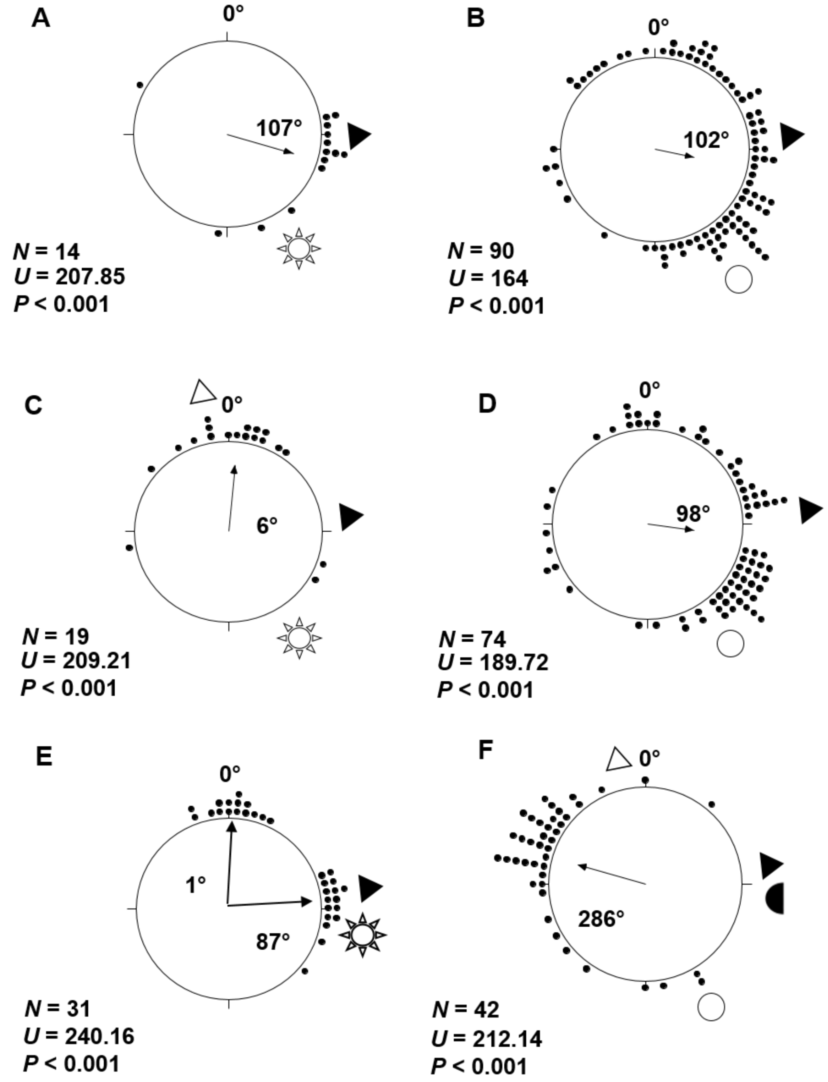

Fig. 3. Animals' entrainment to phase-shifted cycles (Albegna population, zeroed magnetic field). Releases of individuals subjected to an L:D and a lunar cycles in phase with the natural ones under the sun (A) and the moon (B); releases of individuals subjected to an L:D cycle anticipated by 6 hours with respect the natural one under the sun (C) and the moon (D); releases of individuals subjected to a lunar cycle anticipated by 7 days with respect the natural one under the sun (E) and the moon (F). White triangles outside the distributions, expected directions according to the phase-shifting, the symbol of the sun indicates the solar azimuth at the time of tests. The black half-moon corresponds to the theoretical lunar azimuth expected by clock-shifted animals. See Fig. 1 for further explanations.

Discussion

Our results clearly confirm the existence of a chronometric mechanism in *T. europaeus* responsible for the compensation of the azimuthal variations of the moon (Pardi, 1954) on which animals can rely to orientate along the sea-land axis of the beach. In fact, regardless of the lunar azimuth variations, individuals maintain a constant orientation toward the effective landward direction of their home beach. Experiments conducted with the false moon (the electric torch), whose azimuth was arranged differently in respect of that expected, support this statement. Indeed, individuals orientated by assuming an angle to the fake celestial body correspondent to that expected to the natural moon at the time of tests. In the latter case, we adopted the same conditions of illumination used to simulate the false moon in previous studies on sandhoppers facing the same ecological challenge (Ugolini et al., 1999). As stated for *T. saltator* (Ugolini et al., 2002, 2005, 2007; Ugolini, 2003), we can draw first evidence that also in *T. europaeus* the ability to recognise and rely on the moon as a compass cue is not strictly related to the intensity and the spectral composition of the light source.

The use of Earth's magnetic field as an orienting factor is largely widespread among the animal kingdom (Wiltschko & Wiltschko, 1995) and some species of equatorial sandhoppers are known to rely on this cue, especially at times of the day and night when the compensation of the celestial bodies is particularly difficult because of the local astronomical conditions (Pardi et al., 1984, 1988; Ugolini & Pardi, 1992; Ugolini et al., 1999; Ugolini, 2001, 2002, 2016). As previously affirmed, time-compensated lunar orientation is not easy and this aspect can let us speculate that the use of a non-chronometric orienting factor, as the geomagnetic field, can be an effective ecological strategy to overcome the problem. Experiments conducted on *T. europaeus* by Pardi (1954) do not allow us to exclude completely the use of this factor since experimenters operated in conditions of natural magnetic field. A partial evidence was given by the fact that animals modified their orientation when tested under the moon whose azimuth was artificially deflected and orientated properly according to the moon. However, our tests carried out in the absence of the magnetic field or with the magnetic north deflected to the East clearly showed that animals' directional choices were not due to the use of this factor. Even though our tests do not allow us to exclude the capacity of *T. europaeus* to perceive and use the magnetic field in its zonal recovery, we can assess that this species rather relies on the sun or the moon. Our results also indicate that, as demonstrated in *T. saltator* (Ugolini et al., 1999), the entrainment to a clock-shifted L:D cycle affects the solar orientation of *T. europaeus* (individuals deflected their directional choices in accordance with the clock-shifting) while this treatment does not have any effect on its lunar orientation. Instead, the entrainment to a lunar cycle phase-shifted by 7 days seems to affect at least in a part both the sun and the moon compass systems of *T. europaeus*. In fact, in experiments of solar orientation, 48% of the animals tested deflected their directional choices in respect of the expected landward direction in agreement with the moon phase-shifting (by 90° CCW). However, we cannot exclude that the rest (52%) of the individuals, apparently properly orientated toward the landward direction of their home beach, in fact exhibited a positive phototactic tendency since the azimuth of the sun at the time of test (106°) was very close to their escape direction (88°). Anyway, the moon compass system was clearly influenced by the clock-shifting since, in tests of lunar orientation, animals highly deviated from the landward direction of their beach, even though the mean resultant vector recorded is deflected by a great deal (72°) in respect of the new expected direction (358°). In this regard, we would like to underline that, on the basis of the entrainment to a moon cycle anticipated by seven days, the moon phase expected by clock-shifted animals was the last quarter (individuals were in fact tested during the full moon phase). According to the phase-shifting, the moon had still to rise when experiments were conducted (from 2110 hours to 2130 hours) since its rising occurs in the second part of the night over the last quarter phase. Thus, the results obtained represent first evidence that the chronometric mechanism regulating the moon compass probably operates throughout the 24 h period even when the celestial body is not visible. Similarly, in *T. saltator* it has been demonstrated that the moon compass mechanism continues to work even in the absence of the direct vision of the moon (Ugolini et al., 2007) and it is able to assume the correct expected direction all through the lunar month even during the new moon phase.

Our results do not allow us to demonstrate clearly the existence of separate timekeepers involved in the functioning of the two compass systems, as shown on *T. saltator* (Ugolini et al., 1999, 2016). In fact, while the entrainment to a clock-shifted L:D cycle affects only the mechanism of sun compass, the entrainment to a phase-shifted lunar cycle could have an influence on either system. Therefore, further investigations are necessary both to assess the functioning of the moon compass mechanism during the whole lunar month, in particular over the new-moon phase when the astronomical body is not visible, and to clarify the eventual existence of discrete time-keepers regulating the sun and the moon-compass systems.

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CHAPTER 10

GENERAL CONCLUSIONS

The supralittoral sandhopper *T. saltator* constitutes one of the best-known biological models among arthropods for studies of celestial orientation. Since the Fifties, many investigations have shown its capability to use both the sun and the moon in its zonal recovery along the sea-land axis of sandy shores (see Pardi & Ercolini, 1986 for a review). Of course, to rely on celestial cues requires compensatory mechanisms to account for the azimuthal variations exhibited by these factors. Historically, in this species, endogenous time-keepers underlying time-compensated solar and lunar orientation have been investigated through behavioural studies. In particular, it was shown that the oscillator regulating the sun compass system exhibits circadian periodicity (Pardi, 1954; Ugolini et al., 2002) and drives even the rhythm of locomotor activity of this species (Scapini et al., 2005; Ugolini et al., 2007a). In this regard, although overwhelming evidence (Papi & Pardi, 1953, 1954, 1959; Papi, 1960; Enright, 1972), the very existence of a moon compass mechanism in sandhoppers has been long debated (see Wallraff, 1981 for a review); only recently it has been fully confirmed (Ugolini et al., 1999, 2012) and accepted. Further studies showed also that the timing mechanism governing time-compensated lunar orientation in *T. saltator* works throughout the lunar month suggesting its circalunar periodicity (Ugolini et al., 2007b).

Investigations addressed to assess the relationship between the sun and the moon compass systems indicated that discrete oscillators underlie the chronometric compensation of solar and lunar azimuthal variations (Ugolini et al., 1999). In this thesis (chapter 5), the existence of separate oscillators is clearly confirmed, and results obtained indicate a different anatomical localization of the time-keepers regulating the sun and the moon compass mechanisms. Indeed, the surgical removal of antennae disrupted the capability of sandhoppers to orientate to the moon whereas their solar orientation was not affected by this experimental treatment. Moreover, we showed that both first and second antennae are essential to orientate correctly under the moon (chapter 6). Thus, it seems that antennae are the anatomical site of the oscillators responsible for the functioning of the moon compass system whereas those accountable for the compensation of the solar azimuthal variations are located elsewhere. As this regard, taking into account studies indicating the localization of time-keepers that underlie circadian rhythms in other species of crustaceans (see Fanjul-Moles, 2014 for a review), it can be suggested that the mechanism that regulates sun compass-based orientation is governed by coupled oscillators occurring in the central nervous system and its related structures.

Remarkably, the thesis presents the first investigation of endogenous time-keepers in this species from a molecular point of view. Biomolecular analyses (chapter 5) revealed robust circadian oscillations in the expression of the core clock gene homologues *per* and *cry2* (*Talper* and *Talcry2*) in both the brain and the antennae. Since rhythmicity in the expression of clock genes is classically indicative of feedback loops regulating endogenous pace-makers, results presented here provide the first molecular evidence for the existence of time-keepers in *T. saltator*. Furthermore, they could constitute the framework for future studies addressed to assess the functioning of the clock mechanisms of this species.

The rhythmical expression of clock genes observed in the antennae shows that these appendages have the competence for time-keeping and thus support the existence of oscillators located on these appendages. Intriguingly, the circadian expression pattern of the gene *Talcry2* revealed in the antennae is anti-phasic compared to that observed in the brain suggesting that the time-keepers situated in the two anatomical districts exhibit different periodicities. Although further studies are necessary to fully clarify this aspect, results presented here are concordant with the synchronization of the antennal time-keepers to an external cycle other than the 24 h period (putatively the lunar cycle).

The demonstration of antennal time-keepers involved in time-compensated celestial orientation is not without precedent. Indeed, in the Monarch butterfly *D. plexippus* oscillators located in these appendages are responsible for its time-compensated solar orientation (Merlin et al., 2009). In butterflies, it has been also shown that their capability to orientate to the sun is disrupted when time-keepers occurring in one antenna are differently entrained with respect those present in the other antenna (Guerra et al., 2012) indicating a downstream integration of timing information from both appendages.

The thesis provides evidence suggesting that even in *T. saltator* inputs from antennae located on opposite sides of the head undergo a downstream integration in order to generate a robust output signal responsible for the functioning of the moon compass mechanisms. In fact, the unilateral obscuring of these appendages (blocking light-entrainment) disrupted the capability of sandhppers to orientate under the moon (chapter 5). Since the unilateral removal of antennae did not affect the lunar orientation of this species (chapter 6), it was hypothesised that results obtained were due not to a lack of timing inputs but notably to discordant information from oscillators occurring in discrete appendages. Is this regard, it was supposed that photoreceptors in the antennae of this species were eventually responsible for the synchronization of the peripherical time-keepers to the external (lunar) cycle (chapter 8) and electrophysiological investigations clearly revealed electrical responsiveness of the antennae to light stimulation. However, further studies are necessary to elucidate the molecular nature of these receptors, the quality and intensity of light they are stimulated by and their actual involvement in clock-entrainment.

Moreover, in chapter 7, the first localisation of cells within the brain of *T. saltator* expressing core clock genes (*Talper* and *Talpdh*) was achieved through immunological staining. Besides describing the anatomical localization of clock elements, immunological approaches could even point out neuronal connections between different areas of the brain and the antennae implied in the functioning of the biological clocks. In particular, these techniques could facilitate the identification of the anatomical locality where the putative integration of inputs from antennae occurs and reveal eventual relationships between the timing mechanisms underlying the sun and the moon compasses.

Furthermore, results reported in chapter 2 extends the knowledge of the use of the celestial gradients as compass cues by *T. saltator*, following on from the work of Ugolini et al. (2009) who first demonstrated that the skylight luminance gradient constitutes an effective orienting factor for sandhoppers. In natural conditions, the light intensity profile of the sky is coupled with the celestial spectral pattern since both determined by the diffusion of the sunlight through the atmosphere and concordant with the position of the sun. Present work assessed the capabilities of *T. saltator* to use these celestial gradients as individual cues using newly designed artificial sky patterns characterized by the absence of one or the other factor.

Present findings clearly confirm that sandhoppers can rely on the luminance profile of the sky to correctly direct toward their escape direction, indicating that it exists a minimum threshold of the gradient which is recognized and used as an orienting factor by this species. In fact, animals could not perform time-compensated orientation when released under artificial light intensity profiles characterized by luminance variations along the solar meridian attenuated at least by 40% with respect those occurring in clear sky conditions.

The thesis also provided the first evidence for the use of the celestial spectral gradient as a compass cue by *T. saltator*. In fact, sandhoppers released under artificial spectral profiles (in conditions of negligible luminance variations along the solar meridian) at different times of the day exhibited, in any case, effective directional choices toward the escape direction of their home beach. Although it is necessary to refine the experimental procedure in order to reproduce artificial spectral patterns even more similar to that occurring in natural conditions, results clearly demonstrated that even this celestial factor represents an orienting reference for this species.

Experiments of spectral filtering conducted by releasing individuals with discrete regions of their eyes obscured (chapter 3) did not pointed out a clear spatial distribution of their photoreceptors (sensitive to UV-blue and green wavelengths respectively, see Ugolini et al., 2010) typical of other arthropods exhibiting a Dorsal Rim Area (DRA, see Labhart & Meyer, 1999 for a detailed description of this anatomical structure). However, results showed that the dorsal margin of the eye plays an important role in the perception of celestial orienting factors since sandhoppers exhibited increased difficulties to orientate when this region was obscured. Moreover, some morphological differences were observed in the shape of ommatidia located in the dorsal edge and those occurring in the rest of the eye (chapter 4). Indeed, both crystalline cones and rhabdoms are typically hook-shaped in almost all regions of the eye, whereas in the dorsal edge they are straight and thus, according to the anatomical structure of the DRA described for other arthropods (see Labhart & Meyer, 1999 fr a review), potentially suitable to increase polarization sensitivity. Taken together, these results suggest a regionalization of the visual capabilities in this species and are concordant with the existence of a DRA although further studies are necessary to fully elucidate this argument.

Of course, the vision plays a fundamental role in the perception of celestial orienting stimuli by *T. saltator*. Evidence on bent crystalline cones and rhabdoms (chapter 4) apparently indicate a scarce light absorption efficiency of the most part of ommatidia occurring in the compound eye of this species. Indeed, straight optical axis could facilitate the passage of the light through the visual system. However, electrophysiological investigations revealed that these ommatidia exhibit in fact a wide field of view suggesting that reflecting pigment cells, identified between adjacent crystalline cones and retinular cells, could play a role in the enhancement of their photoreception effectiveness.

Finally, results reported in chapter 9 clearly confirm the capability of the isopod *T. europaeus*, syntopic with *T. saltator*, to rely on the moon in its zonal recovery. Since time-compensated lunar orientation has been definitively shown only in few species (see Wallraff, 1981 for a review), results provide a substantial step forward supporting the existence of moon compass-based orientation mechanisms. The data also furnish preliminary evidence for discrete oscillators responsible for time-compensated solar and lunar orientation in coastal species other than *T. saltator*.

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