Keep on track

The use of visual cues for orientation in monarch butterflies

Auf Kurs bleiben

Die Nutzung visueller Hinweise zur Orientierung bei Monarchfaltern

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
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Section Integrative Biology

submitted by

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Summary

The monarch butterfly (*Danaus plexippus*) performs one of the most astonishing behaviors in the animal kingdom: every fall millions of these butterflies leave their breeding grounds in North Amerika and migrate more than 4.000 km southwards until they reach their overwintering habitat in Central Mexico. To maintain their migratory direction over this enormous distance, the butterflies use a time-compensated sun compass. Beside this, skylight polarization, the Earth's magnetic field and specific mountain ranges seem to guide the butterflies as well the south. In contrast to this fascinating orientation ability, the behavior of the butterflies in their non-migratory state received less attention. Although they do not travel long distances, they still need to orient themselves to find food, mating partners or get away from competitors. The aim of the present doctoral thesis was to investigate use of visual cues for orientation in migrating as well as non-migrating monarch butterflies. For this, field experiments investigating the migration of the butterflies in Texas (USA) were combined with experiments testing the orientation performance of non-migratory butterflies in Germany.

In the first project, I recorded the heading directions of tethered butterflies during their annual fall migration. In an outdoor flight simulator, the butterflies maintained a southwards direction as long as they had a view of the sun's position. Relocating the position of the sun by 180° using a mirror, revealed that the sun is the animals' main orientation reference. Furthermore, I demonstrated that when the sun is blocked and a green light stimulus (simulated sun) is introduced, the animals interpreted this stimulus as the 'real' sun. However, this cue was not sufficient to set the migratory direction when simulated as the only visual cue in indoor experiments. When I presented the butterflies a linear polarization pattern additionally to the simulated sun, the animals headed in the correct southerly direction showing that multiple skylight cues are required to guide the butterflies during their migration.

In the second project, I, furthermore, demonstrated that non-migrating butterflies are able to maintain a constant direction with respect to a simulated sun. Interestingly, they ignored the spectral component of the stimulus and relied on the intensity instead. When a panoramic skyline was presented as the only orientation reference, the butterflies maintained their direction only for short time windows probably trying to stabilize their flight based on optic-flow information. Next, I investigated whether the butterflies combine celestial with local cues by simulating a sun stimulus together with a panoramic skyline. Under this conditions, the animals' directedness was increased demonstrating that they combine multiple visual cues for spatial orientation.

Following up on the observation that a sun stimulus resulted in a different behavior than the panoramic skyline, I investigated in my third project which orientation strategies the butterflies use by presenting different simulated cues to them. While a bright stripe on a dark background elicited a strong attraction of the butterflies steering in the direction of the stimulus, the inverted version of the stimulus was used for flight stabilization. In contrast to this, the butterflies maintained arbitrary directions with a high directedness with respect to a simulated sun. In an ambiguous scenery with two identical stimuli (two bright stripes, two dark stripes, or two sun stimuli) set 180° apart, a constant flight course was only achieved when two sun stimuli were displayed suggesting an involvement of the animals' internal compass. In contrast, the butterflies used two dark stripes for flight stabilization and were alternatingly attracted by two bright stripes. This shows that monarch butterflies use stimulus-dependent orientation strategies and gives the first evidence for different neuronal pathways controlling the output behavior.

Zusammenfassung

Der Monarchfalter (*Danaus plexippus*) vollführt eine der atemberaubendsten Verhaltensweisen im Tierreich: Jeden Herbst verlassen Millionen dieser Schmetterlinge ihre Brutgebiete in Nordamerika und migrieren mehr als 4000 km südwärts bis sie ihr Überwinterungsgebiet in Zentralmexico erreichen. Um ihre Migrationsrichtung über diese enorme Distanz einzuhalten, benutzen die Schmetterlinge einen zeitkompensierten Sonnenkompass. Daneben scheinen polarisiertes Licht, das Erdmagnetfeld und bestimmte Gebirgsketten die Schmetterlinge nach Süden zu führen. Im Gegensatz zu dieser faszinierenden Orientierungsfähigkeit wurde dem Verhalten der Schmetterlinge in ihrem nicht-migrierendem Zustand wenig Beachtung geschenkt. Obwohl diese keine großen Distanzen zurücklegen, müssen sie sich dennoch orientieren, um Futter und Paarungspartner zu finden oder Konkurrenten zu entfliehen. Das Ziel der vorliegenden Doktorarbeit war es, die Nutzung visueller Hinweise für die Orientierung von sowohl migrierenden als auch nicht-migrierenden Monarchfaltern zu untersuchen. Dazu wurden Feldexperimente, in denen die Migration der Schmetterlinge in Texas (USA) untersucht wurden, mit Experimenten, in denen das Orientierungsvermögen von nicht-migrierenden Schmetterlingen in Deutschland getestet wurde, verknüpft.

Im ersten Projekt habe ich die Flugrichtung von Schmetterlingen während der jährlichen Herbstmigration aufgezeichnet. In einem Flugsimulator im Freien hielten die Schmetterlinge eine südliche Richtung, solange sie eine freie Sicht auf die Sonne hatten. Eine Versetzung der Sonnenposition um 180° mit Hilfe eines Spiegels zeigte auf, dass die Sonne die wichtigste Orientierungsreferenz der Tiere ist. Des Weiteren konnte ich zeigen, dass die Tiere, wenn die Sonne blockiert und ein grüner Lichtstimulus (simulierte Sonne) eingeschaltet wurde, diese simulierte Sonne als "echte" Sonne interpretierten. Dieser Hinweis reichte jedoch nicht aus, um die Migrationsrichtung festzulegen, wenn er als einziger visueller Hinweis im Labor simuliert wurde. Als ich den Schmetterlingen zusätzlich zur simulierten Sonne ein lineares Polarisationsmuster präsentierte, flogen die Tiere in die richtige, südliche

Richtung. Das zeigt, dass mehrere Himmelshinweise erforderlich sind, um die Schmetterlinge während ihrer Migration zu steuern.

Im zweiten Projekt habe ich weiterhin gezeigt, dass nicht migrierende Schmetterlinge in der Lage sind eine konstante Richtung relativ zu einer simulierten Sonne beizubehalten. Interessanterweise ignorierten sie die spektrale Komponente des Stimulus und verließen sich stattdessen auf die Intensität. Als ein Panorama als einzige Orientierungsreferenz präsentiert wurde, hielten die Schmetterlinge ihre Richtung nur für kurze Zeitfenster und versuchten vermutlich, ihren Flug basierend auf Informationen des optischen Flusses zu stabilisieren. Als Nächstes untersuchte ich, ob die Schmetterlinge Himmelshinweise und lokale Hinweisen kombinieren, indem ich eine Sonne zusammen mit einem Panorama simulierte. Unter diesen Bedingungen war die Gerichtetheit der Flüge erhöht, was zeigt, dass die Tiere mehrere visuelle Hinweise zur räumlichen Orientierung kombinieren.

Beruhend auf der Beobachtung, dass ein Sonnenstimulus zu einem anderen Verhaltensmuster führte als das Panorama, untersuchte ich in meinem dritten Projekt, welche Orientierungsstrategien die Schmetterlinge verwenden. Hierfür präsentierte ich den Tieren verschiedene simulierte Hinweise. Während ein heller Streifen auf dunklem Hintergrund eine starke Anziehungskraft auf die Schmetterlinge, die in die Richtung des Reizes flogen, ausübte, wurde die invertierte Version des Stimulus zur Flugstabilisierung verwendet. Im Gegensatz dazu hielten die Schmetterlinge beliebige Richtungen mit einer hohen Gerichtetheit relativ zu einer simulierten Sonne ein. In einer uneindeutigen Szenerie mit zwei identischen Reizen (zwei helle Streifen, zwei dunkle Streifen oder zwei Sonnenstimuli), die um 180° versetzt waren, wurde eine konstante Flugrichtung nur dann erreicht, wenn zwei Sonnenstimuli gezeigt wurden. Das deutet auf eine Beteiligung des inneren Kompasses der Tiere hin. Im Gegensatz dazu nutzten die Schmetterlinge zwei dunkle Streifen zur Flugstabilisierung und wurden abwechselnd von zwei hellen Streifen angezogen. Dies zeigt, dass Monarchfalter stimulus-abhängige Orientierungsstrategien verwenden, und liefert den ersten Nachweis für unterschiedliche neuronale Verschaltungswege, die das Verhalten steuern.

Contents

1 General introduction

1.1 Animal orientation

For most animals locomotion is essential to survive and thrive in their habitat. The motivation behind maneuvering from one place to another can be extremely manifold including escaping harmful conditions, searching for new food sources or finding mating partners. In a number of these situations moving in a constant direction is the most efficient strategy and therefore being able to orientate in the environment is essential. Orientation in space requires animals to control their body axis in relation to their surrounding environment. The longer animals travel in one direction the more complex the underlying orientation strategies become to minimize the accumulation of errors. One very simple strategy is a fast and small movement away from a looming stimulus helping a fly to escape a predator (Card, 2012). Other animals use a fixation behavior when perceiving stimuli that are attractive to them, such as the odor of a food source (Buehlmann et al., 2014) or the released pheromone or calling song of a mating partner (Hansson, 1995; Schmitz et al., 1982), or an anti-fixation to get away from aversive stimuli (Maimon et al., 2008). Flies can even switch between these behaviors flying away from a small object they might interpreted as a predator and moving towards a larger object which can serve as a landing site (Ache et al., 2019; Maimon et al., 2008). These anti-fixation and fixation are rather simple strategies to cover short distances in a defined direction in relation to a detectable target (towards or away from it). In contrast to this, menotaxis describes a behavior of animals moving in an arbitrary direction by maintaining a specific angle relative to a reference point (Beetz and el Jundi, 2018; Giraldo et al., 2018). After forming a ball, dung beetles for example display this fascinating behavior by rolling their food in a straight line away from the dung pile to avoid competitors (Baird et al., 2010a; Dacke and el Jundi, 2018; Dacke et al., 2011; Dacke et al., 2013a; Dacke et al., 2013b). An even more complex strategy is path integration performed by some insects showing incredible abilities to find their way from a food source back home. As central place foragers, desert ants leave their nest to travel large distances until they find food. During this outbound movements they constantly integrate information about the distance and travel direction (Collett and

Collett, 2000; Pfeffer and Wittlinger, 2016; Ronacher and Wehner, 1995; Wittlinger et al., 2006), enabling them to choose the shortest way back to the nest (Müller and Wehner, 1988). Beside these forms of orientation, one seasonal occurrence has been fascinating humans for decades: long-distance migration. When environmental factors like food availability or temperature become unfavorable, some animals, including birds (Hiemer et al., 2018; Visser et al., 2009) and bats (Fleming, 2019), leave their habitats and migrate over thousands of kilometers to more suitable locations. Apart from vertebrates, many insects display an annual migration cycle. The Australian Bogon moth, for example, travels up to 1000 km from southern Queensland, western and northwestern New South Wales, and western Victoria to caves in New South Wales and Victoria for hibernation (Warrant et al., 2016). These moths as well as European moths such as the red underwing and the large yellow underwing migrate during the night to escape lethal temperatures (Dreyer et al., 2018b). In contrast to this, other lepidopteran like the North American monarch butterfly were found to migrate during the day time (Reppert et al., 2016) pursuing the same goal.

One question fascinating neuroethologists for many decades is how insects in particular, with their brains smaller than a grain of rice, control stable orientation behaviors from fixation/anti-fixation and menotaxis to path integration and long-distance migration.

1.2 Maintaining a directed course

For all orientation behaviors, migration, path integration or straight-line orientation during dispersal, maintaining a directed course is crucial. To achieve this, animals need to control their body axis in relation to their surrounding environment. For this, they can rely on different orientation references provided by external cues from the environment or internal reference systems such as self-motion. Over short distances or time windows, one cue can be sufficient to guide an animal along a straight path. However, the longer the route gets, the more errors accumulate and therefore a single orientation reference is less reliable. To overcome this problem and maximize an animal’s survival success, many animals do not rely exclusively on one cue but dynamically switch between them (el Jundi et al., 2019; Lebhardt and Ronacher, 2015; Patel and Cronin, 2020; Wehner, 1997) or combine them for orientation (Dacke et al., 2019; Dreyer et al., 2018a; el Jundi et al., 2014a).

1.2.1 External cues

When setting a specific direction towards a goal, external cues provide a reliable reference system. Depending on the distance, these cues can be perceived using different sensory modalities. Female crickets (*Gryllus campestris* L.) perform phonotaxis following the calling songs of male individuals in a straight line (Schmitz et al., 1982). Beside these acoustic signals, odor cues can be used over short distances to find food (Buehlmann et al., 2014), a nest entrance (Steck et al., 2009), or mating partners that release pheromones (Hansson, 1995). In addition, for the majority of animals, vision plays a key role for orientation. At familiar places, specific landmarks can be used to find the correct way. Central place forager, such as bees and ants, that return to the nest after a successful foraging trip can pinpoint their home by comparing their visual surroundings to nest-associated landmarks (Brünnert et al., 1994; Fleischmann et al., 2016) which they learned during orientation flights/walks (Fleischmann et al., 2018a; Zeil et al., 1996). But when animals leave their familiar area, traveling through places they never visited before, they can no longer rely on landmarks due to the lack of information about their relation between the landmarks and the animals' goal. To maintain a specific direction under this condition, animals can still rely on visual information: celestial cues. Independent from the location on earth, celestial cues provided by the sun or moon offer directional information. During the day, the sun is the most prominent orientation reference and easily detectable as the brightest spot in the sky. Its position is defined by the solar azimuth and elevation (Fig. 1A) and found to be the most important reference for orientation in many diurnal insects. To disperse from a dung pile, dung beetles were found to maintain an arbitrary direction relative to the sun (Baird et al., 2010a; Dacke et al., 2014; Khaldy et al., 2020). In path integrating arthropods such as ants, bees, and mantis shrimps, the position of the sun is the basis of their sun-compass system guiding them home (Lebhardt and Ronacher, 2015; Patel and Cronin, 2020; von Frisch and Lindauer, 1956; Wehner, 1997). Even more, were bees found to communicate the location of profitable food sources in their waggle dance in relation to the sun’s position (von Frisch and Lindauer, 1956). However, the sun’s azimuth changes over the course of the day and bees dancing over a longer time period were found to correct their dance orientation with the sun’s movement (von Frisch and Lindauer, 1956). This clearly shows that bees have knowledge about the course of the sun and the time of the day to make their sun compass time compensated.

Apart from its position, the sun offers secondary cues (polarization, color, and brightness) that can be used for orientation. Light emitted by the sun is white, containing all wavelength, and unpolarized until it enters the atmosphere where it gets scattered by air molecules changing the lights’ color, intensity, and oscillation. Thereby, the light becomes partly linearly polarized and a pattern of polarized light is created building concentric circles around the sun (Fig. 1B). Depending on the position of the sun, the angle and degree of polarization changes and reaches its’ maximum of about 70% degree of polarization perpendicular to the sun (Horváth and Wehner, 1999; Wehner, 2001). The resulting pattern of polarized light, therefore, provides directional information closely related to the sun’s position. In contrast to humans, many insects possess a specialized eye region to perceive this information. In the so-called dorsal rim area (DRA), the microvilli along the rhabdomere are arranged in one line, each ommatidium containing two sets of photoreceptors with a 90° microvilli orientation to each other (Labhart and Meyer, 1999). This physiological adaptation allows the perception of polarized light and enables insects such as bees, ants, dung beetles, and flies to obtain directional information for orientation (el Jundi et al., 2014a; von Frisch, 1949; Warren et al., 2018; Wehner, 1997). Some animals are even able to transfer information between the polarization compass and the sun compass (Lebhardt and Ronacher, 2015; Wehner, 1997). Additional to the skylight polarization, the scattering of sunlight results in celestial gradients. While short wavelengths are uniformly distributed, the distribution of longer wavelengths depends on its azimuthal distance. This means that in the direction of the sun a higher amount of long wavelength is found while a relatively higher amount of short wavelength refers to the anti-solar hemisphere (Coemans et al., 1994; el Jundi et al., 2015a; Fig. 1C). The chromatic contrast is thereby directly connected to the position of the sun where it reaches its maximum (Coemans et al., 1994). Some insects, including bees and dung beetles use this type of information for orientation (Brines and Gould, 1979; Edrich et al., 1979; el Jundi et al., 2015a; Rossel and Wehner, 1984). Directly linked to the chromatic contrast, the total intensity of light differs between the brighter solar and dimmer anti-solar hemisphere. Together with information from polarized light, this celestial cue can help dung beetles to steer straight (el Jundi et al., 2014a).

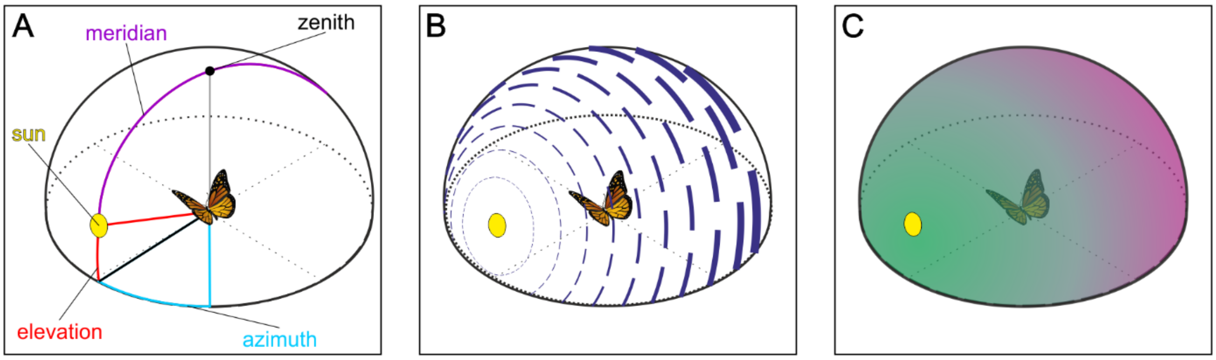


Fig. 1: Celestial cues provide directional information. (A) The position of the sun (yellow) is defined by its elevation (red) and azimuth (blue). Over the course of the day the sun moves along the meridian (purple) passing the zenith (black). (B) Light scattered by atmospheric molecules gets partly polarized building concentric circles around the sun (yellow circle). The pattern of polarized light is defined by its angle (direction of the blue lines) and degree of polarization (indicated by the thickness of the blue lines). Modified from Nguyen et al. (2021). (C) As light of different wavelengths gets scattered differently, a spectral contrast is generated in the sky. While short wavelength (purple) is distributed uniformly, light of longer wavelength (green) gets scattered non-uniformly. Thereby, the relative amount of shorter wavelength is higher in the antisolar hemisphere, while the amount of longer wavelength is higher in the solar hemisphere. Modified from Nguyen et al. (2021).

1.2.2 Optic flow and idiothetic cues

Insects can rely on a number of different external cues to set their desired heading in the environment and especially visual cues offer a good reference for orientation. But how can they maintain their course and compensate involuntary disturbances? When flies were tested in a homogeneous visual environment, a grating pattern of black and white stripes, they were able to maintain a straight flight and follow a rotation of the visual scenery (Wolf and Heisenberg, 1990). This is a simple optomotor response for flight stabilization that was additionally found in flying locusts (Robert, 1988) and hoverflies (Collett, 1980). Whenever an animal is moving in space, the image of the surrounding environment is shifting in the opposite direction, producing optic-flow information. Thereby, translational optic flow resulting from forward or backward movements is used to control flight speed (Baird et al., 2005; Baird et al., 2010b), an animal's position in enclosed environments (Baird et al., 2010b; Kirchner and Srinivasan, 1989; Stöckl et al., 2019), or estimate traveled distances (Ronacher and Wehner, 1995; Srinivasan et al., 2000). Apart from this, sidewards translational optic flow from movements to the side and rotational optic flow from a turn in the animals' yaw axis are used for flight stabilization. For this and to maintain an intended direction, animals compensate involuntary movements or disturbances using optic flow by minimizing the image movements (Srinivasan and Zhang, 2004).

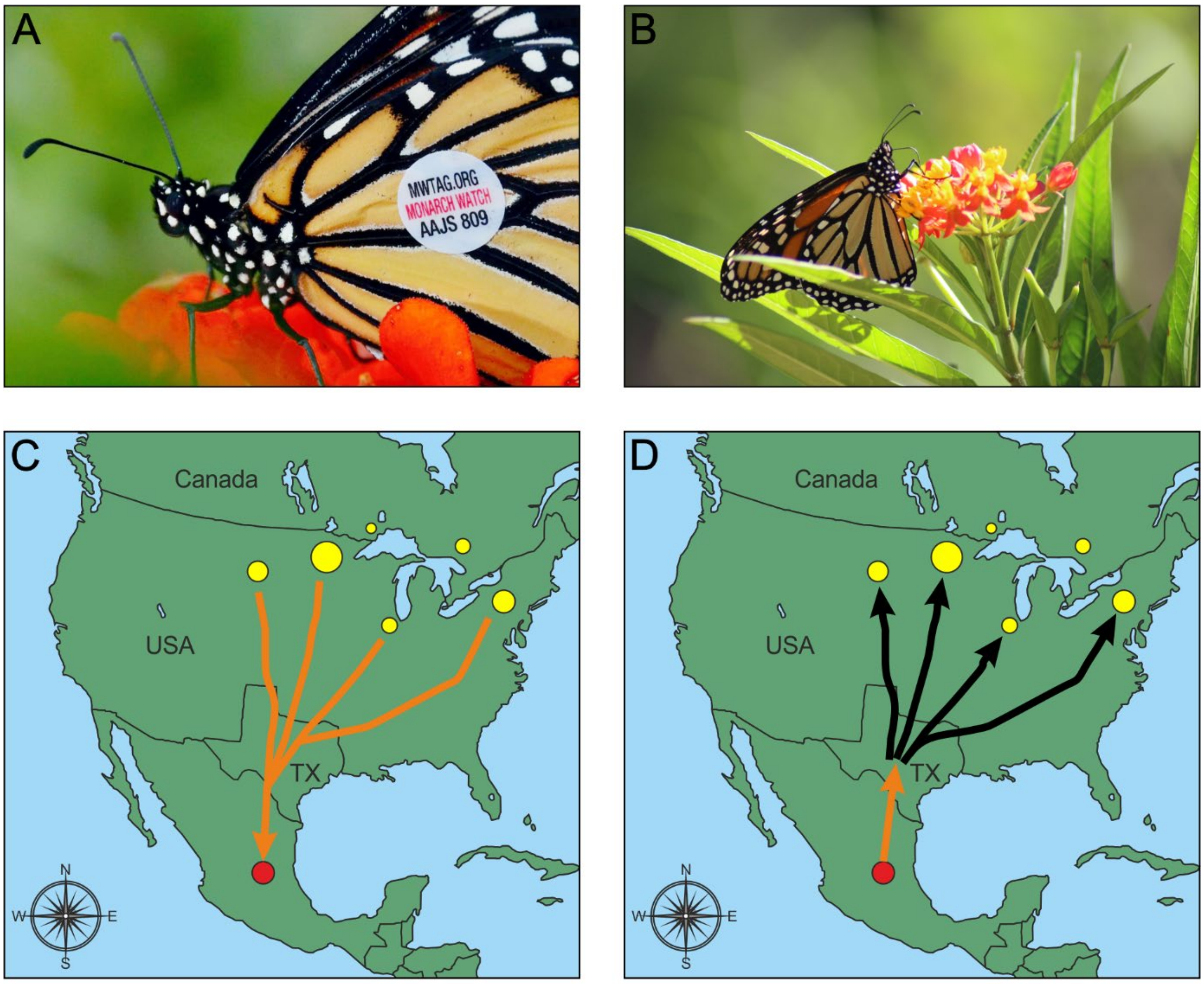
In contrast to involuntary movements, the animal is gaining idiothetic cues as self-motion signals from proprioceptive feedback, mechano-sensory information, and motor efference copies (Varga et al., 2017) from voluntary turns. In the fruit fly *Drosophila*, idiothetic cues are used to perform a centered search at a food source they previously established (Kim and Dickinson, 2017) and to maintain a directed course towards a visual goal that disappeared during approaching (Neuser et al., 2008; Strauss and Pichler, 1998). Neuronal studies revealed that these behaviors are enabled via an internal representation of the animals' body axis in the environment (Kim et al., 2019; Lu et al., 2021; Lyu et al., 2021) that consist even in complete darkness (Seelig and Jayaraman, 2015; Turner-Evans et al., 2017) in the brain of *Drosophila*. A similar heading-direction network exists in the monarch butterflies' brain and was most recently found to integrate idiothetic cues (Beetz et al., 2021). This insect displays the fascinating behavior of a long-distance migration and acts as a perfect model organism to study orientation.

1.3 Monarch butterflies

The annual migration of the eastern North American monarch butterfly (*Danaus plexippus*) is one of the most spectacular phenomena in insect orientation. Hundreds of US citizens teach their children about this fascinating long-distance migration, build butterfly-friendly gardens, raise, release, tag, and monitor 'their own monarchs' (Fig. 2A; https://monarchwatch.org/) to counteracts the ongoing declining in the butterflies' populations (Semmens et al., 2016; Thogmartin et al., 2017).

1.3.1 The migration cycle

During the summer, monarch butterflies breed in North America and the southern parts of Canada where they are often found in close proximity to milkweed (Fig. 2B). These host plants of the genus *Asclepias* are the primary food source of the butterflies' food specialized caterpillars. However, in the late summer and early fall the food quality starts to decrease and together with shorter daylength and declining temperatures the onset of the annual migration cycle is triggered (Goehring and Oberhauser, 2002) with a new generation, the fall generation, of monarch butterflies hatching. These migratory individuals genetically differ from the summer population by possessing larger fat bodies, higher immune responses, and longer lifespans (Zhu et al., 2009) to facilitate a successful migration. In comparison to the non-migratory individuals, the fall generation is in a sexual diapause caused by a reduced juvenile hormone level (Zhu et al., 2009), which enables the butterflies to save energy for the long journey. Every fall, millions of these colorful insects leave their breeding grounds and migrate over more than 4.000 km southwards until they reach their overwintering site in specific oyamel fir groves in the Mountains of Michoacán in Central Mexico (Urquhart and Urquhart, 1976; Fig. 2C). In these forests, the adult butterflies hibernate in roosts until spring, when cold exposure triggers the onset of the north migration (Guerra and Reppert, 2013). The same generation of butterflies that displayed the south migration now become fertile and start to return to their breeding grounds (Fig. 2D orange arrow). On their route, these butterflies lay their eggs on freshly growing milkweed plants and the subsequent generations repopulate the breeding sides fulfilling the multi-generational migration (Fig. 2D black arrows).



fall migration

spring migration

Fig. 2: The annual migration cycle of the eastern North American monarch butterfly. (A) To unravel the migration route and find the overwintering habitat of the butterflies, individuals were equipped with small tags on their lower wing. Tagging events take place every fall to monitor the population of monarch butterflies successfully arriving in their overwintering area. Photo credit: MonarchWatch. (B) A monarch butterfly feeding on its host plant of the genus Asclepias. (C) In the summer, monarch butterflies can be found in different populations (yellow circles) in the northern part of the United States and South Canada. During the fall migration, a migratory generation (orange arrows) maintain a southerly direction more than 4.000 km, passing Texas (TX) to reach their overwintering site in Central Mexico (red dot). (D) In spring, the same individuals (orange arrow) start the spring migration. These butterflies lay their eggs on fresh milkweed and the subsequent generations (black arrows) repopulate the breeding sides (yellow circles).

1.3.2 Orientation abilities

During the fall, behavioral experiments in flight simulators reveled that monarch butterflies possess a sun compass and rely on the position of the sun to migrate southwards (Froy et al., 2003; Heinze and Reppert, 2011; Mouritsen and Frost, 2002; Reppert, 2006). The sun's azimuth changes over the course of the day and if the butterflies keep the same angle relative to it, they would not be able to maintain their southerly migratory direction (Fig. 3 upper panel). Experiments time-shifting the animals in incubators demonstrated that depending on the time of day, the butterflies adjust their angle to the sun (Froy et al., 2003; Mouritsen and Frost, 2002) by integrating time information of their antennal circadian clocks (Merlin et al., 2009). This makes their sun compass time compensated and guides them towards the South (Fig. 3 lower panel).

Studies investigating the photoreceptors of monarch butterflies reported that these animals possess a DRA containing approximately 100 ommatidia (Labhart et al., 2009) sensitive to polarized light in the UV spectrum (Sauman et al., 2005; Stalleicken et al., 2006). Together with electrophysiological findings (Heinze and Reppert, 2011; Nguyen et al., 2021), this demonstrates that the butterflies can perceive polarized light. However, behaviorally investigations testing the use of the angle of polarized light for orientation in monarch butterflies under a linear polarization filter led to contradicting results. In one study, the butterflies followed a 90° rotation of the polarizers' axis (Reppert et al., 2004) while in another study they did not (Stalleicken et al., 2005). The authors of this latter study furthermore hypothesized that the butterflies rather use celestial gradients to keep their migratory direction when the direct view of the sun is blocked (Stalleicken et al., 2005). However, this hypothesis is not confirmed yet.

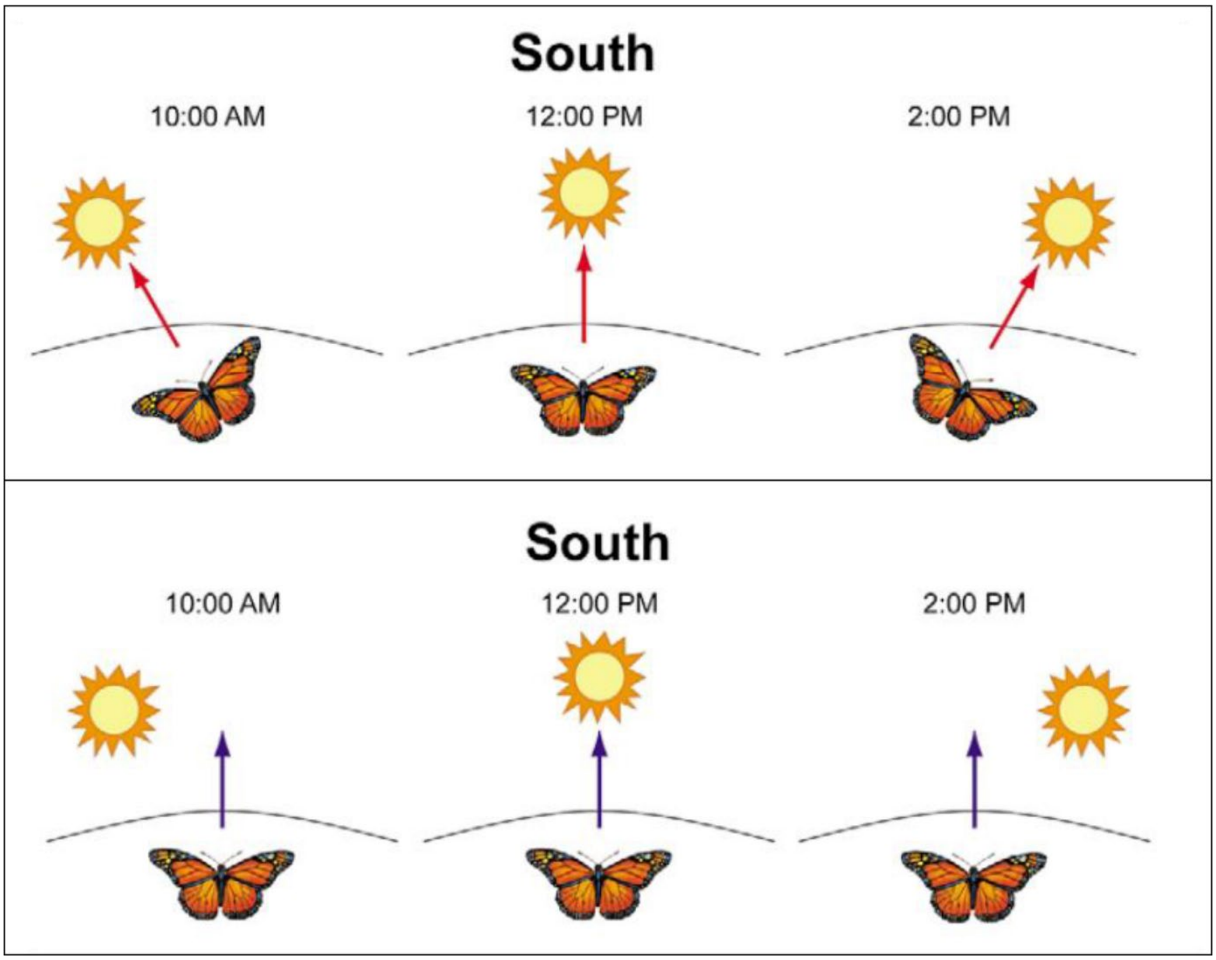


Fig. 3: The time-compensated sun compass of monarch butterflies. Monarch butterflies compensate for the movement of the sun. Upper panel: if butterflies maintain the same angle relative to the sun, the animals were not able to keep the southerly migratory direction. Lower panel: combining time-of-day information with the position of the sun, monarch butterflies compensate for the sun's movement to maintain their course towards the South. Modified from Reppert (2007).

1.3.3 Dispersal behavior

While the interest of research on monarch butterflies focuses on the annual migration and especially on the southerly fall migration, the behavior of the non-migrating generations is only poorly understood. Observations in the butterflies' breeding grounds revealed that they mainly display behaviors associated with feeding, mating and oviposition (Calvert, 2001). In comparison to the migratory generation, the non-migratory butterflies perform only short flights maintaining a directed course with each individual preferring a different direction (Calvert, 2001; Zhu et al., 2009). This results in dispersal behavior, a common strategy in Lepidopterans (Felt, 1925; Stevens et al., 2010). On a smaller scale, dispersal can be described as spreading away from each other, away from the place of origin, or a high density of animals (Stevens et al., 2010) to increase the individual's success in finding food and mating partners by escaping competitors. Dispersal is also observed on a larger geographical scale driving gene flow in new location (Ronce, 2007) by a population trying to find a new niche. On the basis of such a dispersal, populations of monarch butterflies are not restricted to North America but also found in Central and South America as well as Australia and the Caribbean (Merlin and Liedvogel, 2019; Zalucki and Clarke, 2004; Fig. 4). Interestingly, the butterflies of these habitats are genetically separated from the North American population and do not develop a migratory generation, except of the Australian population (Zhan et al., 2014).

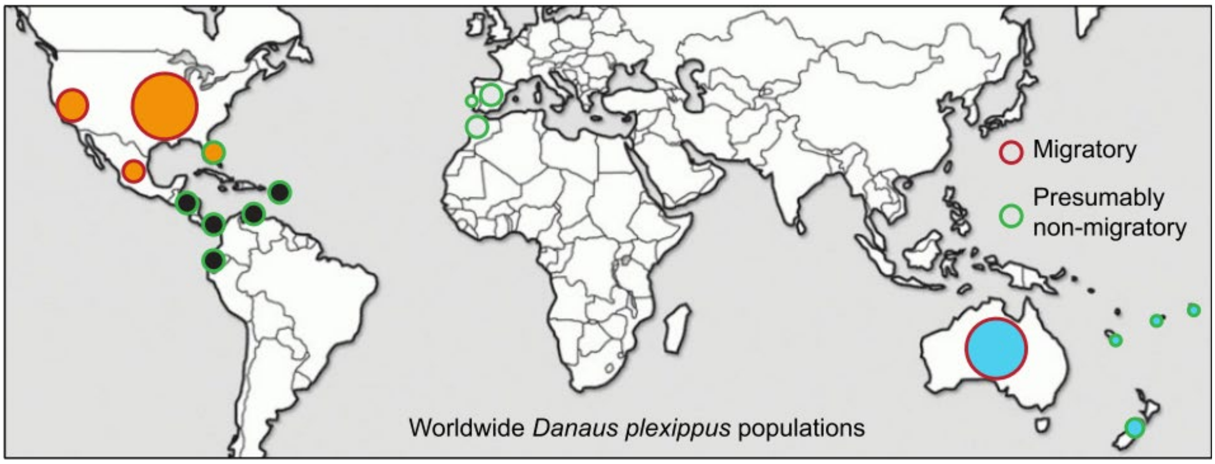


Fig. 4: Worldwide populations of monarch butterflies. While only butterflies in North America and Australia were observed to undergo an annual migration cycle (circles with red border), the majority of monarch butterflies in South and Central America as well as the Caribbean is non-migratory (circles with green borders). Genetic analysis revealed the similarity of the genetic structure between the different populations (indicated by the colors within the cycles). Adapted from Merlin and Liedvogel (2019).

1.4 The monarch butterflies' brain

Despite its small size, the brain of an insect is complex and plastic in its neuroarchitecture and function (Chittka and Niven, 2009). Multiple cues arriving from different, or the same sensory input, must be integrated and weighted to display an adequate behavior. This output must constantly be controlled and adjusted by evaluating sensory feedback. Electrophysiological and neuroanatomical investigations help us to understand the structure and operating principles of the highly organized insect brain. For the monarch butterfly the central brain with its neuronal connections was 3D reconstructed (Heinze and Reppert, 2012; Heinze et al., 2013) to generate a brain atlas and compare it with other insect species (Heinze et al., 2021) such as dung beetles (Immonen et al., 2017), bumble bees (Rother et al., 2021), and desert ants (Habenstein et al., 2020). This illustrated the large size of the butterflies' optic lobes, in total about 75% of the whole brain volume (Fig. 5A), (Heinze and Reppert, 2012) giving evidence that vision plays an important role for these insects. Further comparing the neuropiles between species such as flies, locusts, dung beetles, and monarch butterflies, many brain regions including the central complex (CX; Fig. 5A&B) were found to be highly conserved (el Jundi et al., 2018; Hanesch et al., 1989; Heinze and Homberg, 2008). This midline spanning neuropile is divided in the upper and lower division of the central body, the protocerebral bridge, and the paired noduli (Heinze and Reppert, 2012; Heinze et al., 2013; Fig. 5B). The CX is the center for sensory integration and motor control in insects (Homberg, 2008; Strauss, 2002) and houses the heading-direction network (Fisher et al., 2019; Green et al., 2017; Kim et al., 2019; Lu et al., 2021; Lyu et al., 2021; Seelig and Jayaraman, 2015; Stone et al., 2017; Turner-Evans et al., 2017) controlling goal-directed movements in *Drosophila* and monarch butterflies (Beetz et al., 2021). The most prominent role of the CX is its function as the animals internal compass (el Jundi et al., 2014b; Heinze and Reppert, 2011; Heinze et al., 2013; Pfeiffer and Homberg, 2014) and its function has been highly investigated in the locust (el Jundi and Homberg, 2010; Heinze and Homberg, 2008; Homberg, 2004; Homberg et al., 2011; Kinoshita et al., 2007; Pegel et al., 2018) and more recently in the monarch butterfly (Beetz et al., 2021; Heinze and Reppert, 2011; Heinze et al., 2013; Nguyen et al., 2021). Compass-related information, such as the angle of polarized light and the position of the sun (Beetz et al., 2021; Heinze and Reppert, 2011; Nguyen et al., 2021), is perceived by photoreceptors of the DRA and the remaining eye and transferred throughout the optic lobes to the anterior optic tubercles. From here, the input is sent to the bulbs before entering the CX (Heinze et al., 2013). Neurons of the CX in monarch butterflies show a response depending on the angle of polarized light and encode the azimuthal position of the sun (Heinze and Reppert, 2011; Nguyen et al., 2021). After integration in the CX, output information is sent to the lateral accessory lobe and further to the posterior protocerebrum (Heinze et al., 2013). In this circuit, the animals' behavioral state plays an important role. While a sun-bearing coding was found in resting butterflies, the heading-direction network integrates idiothetic cues when the animals are flying to encode the compass representation (Beetz et al., 2021).

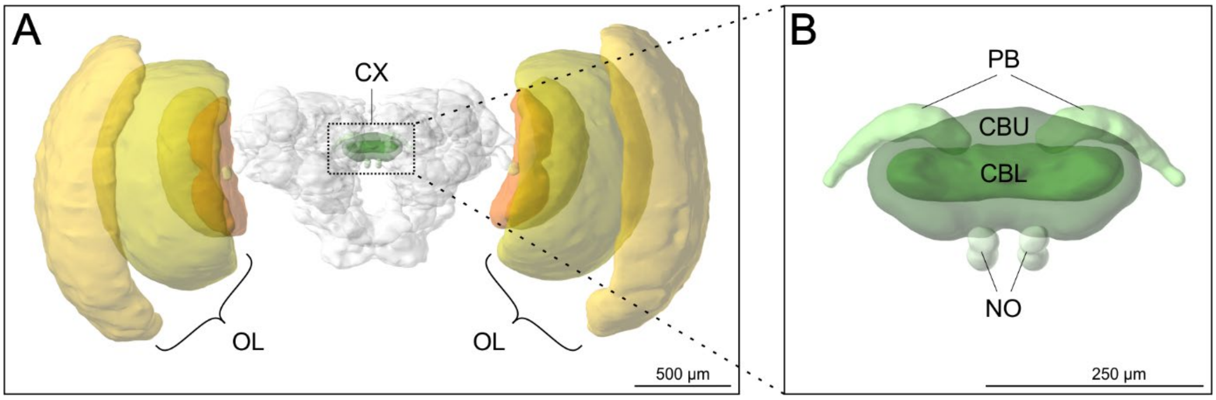


Fig. 5: The central brain of the monarch butterfly. (A) Frontal view of the standardized butterfly brain with their large optic lobes (OL) highlighted in yellow and the central complex (CX) highlighted in green. Scale bar: 500 µm. (B) Enlargement of the central complex consisting of the protocerebral bridge (PB), the upper (CBU) and lower division of the central body (CBL) and the noduli (NO). Scale bar: 250 µm. The brain and central complex of the monarch butterfly is adapted from Heinze and Reppert (2012) and created via https://insectbraindb.org (Heinze et al., 2021).

2 Thesis Outline

The monarch butterfly is a prominent model organism to study long-distance migration. The spectacular annual migration between its breeding ground in North America and South Canada and the overwintering site in Central Mexico fascinates humans since decades. These colorful butterflies maintain a southerly direction over more than 4.000 km throughout unfamiliar places they never visited before. Although many studies investigate the southerly fall migration, still little is known about underlying mechanisms of the butterflies' orientation strategies. While it has been shown that the animals rely on a time-compensated sun compass (Froy et al., 2003; Mouritsen and Frost, 2002) and sense the geomagnetic field (Guerra et al., 2014; Wan et al., 2021), the use of other cues such as polarized light (Reppert et al., 2004; Stalleicken et al., 2005) and landmarks (Calvert, 2001) remains unclear. Furthermore, only few studies focus on the orientation of the butterflies in the non-migratory state. Although the distance they travel is much smaller, they still need to orient to find food, mating partners and avoid predators and competitors by dispersal behavior. The aim of this thesis is to answer the questions:

- Which orientation strategies do monarch butterflies display?
- Which cues do monarch butterflies use for orientation?
- Which cues do monarch butterflies rely on during their annual fall migration?
- How to monarch butterflies weigh different cues?

In this thesis I will address these questions in the following three chapters:

1. The use and hierarchy of celestial cues in migrating monarch butterflies

In this first chapter, the use of different celestial cues (sun, polarized light and celestial gradients) during the southerly migration of the butterflies was investigated in a series of flight simulator experiments. This study was performed on wild-caught monarch butterflies that passed Texas (USA) during their fall-migration by either presenting the animals the natural sky or single simulated cues under very controlled laboratory conditions indoors. Additionally, cue-conflict experiments were conducted to reveal the underlying hierarchy of the different orientation references the butterflies relied on.

2. Spatial orientation cues in non-migrating monarch butterflies

Orientation does not only play a major role during the migration of butterflies. Also, the non-migrating population of butterflies need to find their way in their environment. In this chapter, we focused on the question whether non-migrating monarch butterflies combine multiple visual cues for spatial orientation. In an 360° LED flight simulator we first presented the animals either simulated sun stimuli with different spectral information or a simulated panoramic skyline for orientation. In a next step, we combined both cues investigating the effect on the butterflies' performance.

3. Orientation strategies in monarch butterflies

To maintain a directed course based on visual stimuli, animals can rely on different orientation strategies either facing directly towards a stimulus, keeping a certain angle to it, or stabilizing the produced optic flow when moving in space. The last chapter focuses on these different types of orientation in monarch butterflies by presenting different stimuli to the tethered animals. The heading directions of butterflies were recorded in flight simulators while the animals perceived either simulated sun stimuli or stripes of different contrast. Using an ambiguous scenery, we investigated if the butterflies take both cues as the same or combine them for compass orientation. Here we aimed to understand how the behavioral outcome is controlled and which brain regions are likely involved in controlling them.

Manuscript 1

Multiple skylight cues guide monarch butterflies along their migratory route

Myriam Franzke, Tu Anh Thi Nguyen, Christine Merlin, Basil el Jundi

Abstract

Each fall millions of monarch butterflies (*Danaus plexippus*) migrate over thousands of kilometers from North America to their overwintering habitat in Central Mexico. To maintain their southerly direction over this enormous distance, these butterflies rely on the sky for orientation. But which celestial cues do they use exactly and which of them are essential to set the migratory direction? To investigate this, we tested migratory monarch butterflies while they were tethered at the center of a flight simulator and were able to freely change their bearing with respect to a visual scene. We first studied the butterflies in a flight simulator outdoors and found that they kept constant southward directions under the natural sky. When we then displaced the sun by 180° using a mirror, most animals changed their heading direction by about 180°. This suggests that the sun acts as their main migratory cue. We then performed indoor experiments and presented the green light spot, representing a mimicked sun, as the only source of orientation reference to the butterflies. Interestingly, instead of heading in the correct migratory direction, the animals exhibited arbitrary heading directions, which indicates that the sun is not a sufficient reference to set the migratory direction. We next tested the butterflies under a linear polarization filter outdoors and found that they use polarized light in combination with other skylight cues as orientation reference. When we presented the mimicked sun in combination with a simulated polarized skylight to the butterflies indoors, the animals kept constant flight directions that accurately matched the migratory directions in nature. These findings demonstrate that the sun and polarized light are essential cues to guide the monarch butterflies on their long journey to Central Mexico.

Manuscript 2

Spatial orientation based on multiple visual cues in non-migratory monarch butterflies

Myriam Franzke, Christian Kraus, David Dreyer, Keram Pfeiffer, M. Jerome Beetz, Anna L. Stöckl, James J. Foster, Eric J. Warrant, Basil el Jundi

Journal of Experimental Biology (2020) 223: 1-12

Abstract

Monarch butterflies (*Danaus plexippus*) are prominent for their annual long-distance migration from North America to their overwintering area in Central Mexico. To find their way on this long journey, they use a sun compass as their main orientation reference but will also adjust their migratory direction with respect to mountain ranges. This indicates that the migratory butterflies also attend to the panorama to guide their travels. While the compass has been studied in detail in migrating butterflies, little is known about the orientation abilities of non-migrating butterflies. Here we studied if non-migrating butterflies - that stay in a more restricted area to feed and breed - also use a similar compass system to guide their flights. Performing behavioral experiments on tethered flying butterflies in an indoor LED flight simulator, we found that the monarchs fly along straight tracks with respect to a simulated sun. When a panoramic skyline was presented as the only orientation cue, the butterflies maintained their flight direction only during short sequences suggesting that they potentially use it for flight stabilization. We further found that when we presented the two cues together, the butterflies incorporate both cues in their compass. Taken together, we here show that non-migrating monarch butterflies can combine multiple visual cues for robust orientation, an ability that may also aid them during their migration.

Manuscript 3

Stimulus-dependent orientation strategies in monarch butterflies

Myriam Franzke, Christian Kraus, Maria Gayler, David Dreyer, Keram Pfeiffer, Basil el Jundi

Journal of Experimental Biology (2022) in press

Abstract

Insects are well-known for their ability to keep track of their heading direction based on a combination of skylight cues and visual landmarks. This allows them to navigate back to their nest, disperse throughout unfamiliar environments, as well as migrate over large distances between their breeding and non-breeding habitats. The monarch butterfly (*Danaus plexippus*) for instance is known for its annual southward migration from North America to certain trees in Central Mexico. To maintain a constant flight route, these butterflies use a time-compensated sun compass for orientation which is processed in a region in the brain, termed the central complex. However, to successfully complete their journey, the butterflies' brain must generate a multitude of orientation strategies, allowing them to dynamically switch from sun-compass orientation to a tactic behavior toward a certain target. To study if monarch butterflies exhibit different orientation modes and if they can switch between them, we observed the orientation behavior of tethered flying butterflies in a flight simulator while presenting different visual cues to them. We found that the butterflies' behavior depended on the presented visual stimulus. Thus, while a dark stripe was used for flight stabilization, a bright stripe was fixated by the butterflies in their frontal visual field. If we replaced a bright stripe by a simulated sun stimulus, the butterflies switched their behavior and exhibited compass orientation. Taken together, our data show that monarch butterflies rely on and switch between different orientation modes, allowing the animal to adjust orientation to its actual behavioral demands.

3 Manuscript I:

Multiple skylight cues guide monarch butterflies along their migratory route

Multiple skylight cues guide monarch butterflies along their migratory route

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Abstract

Each fall millions of monarch butterflies (*Danaus plexippus*) migrate over thousands of kilometers from North America to their overwintering habitat in Central Mexico. To maintain their southerly direction over this enormous distance, these butterflies rely on the sky for orientation. But which celestial cues do they use exactly and which of them are essential to set the migratory direction? To investigate this, we tested migratory monarch butterflies while they were tethered at the center of a flight simulator and were able to freely change their bearing with respect to a visual scene. We first studied the butterflies in a flight simulator outdoors and found that they kept constant southward directions under the natural sky. When we then displaced the sun by 180° using a mirror, most animals changed their heading direction by about 180°. This suggests that the sun acts as their main migratory cue. We then performed indoor experiments and presented the green light spot, representing a mimicked sun, as the only source of orientation reference to the butterflies. Interestingly, instead of heading in the correct migratory direction, the animals exhibited arbitrary heading directions, which indicates that the sun is not a sufficient reference to set the migratory direction. We next tested the butterflies under a linear polarization filter outdoors and found that they use polarized light in combination with other skylight cues as orientation reference. When we presented the mimicked sun in combination with a simulated polarized skylight to the butterflies indoors, the animals kept constant flight directions that accurately matched the migratory directions in nature. These findings demonstrate that the sun

and polarized light are essential cues to guide the monarch butterflies on their long journey to Central Mexico.

Introduction

To escape unfavorable environmental conditions, many animals display the astonishing phenomenon of an annual long-distance migration. One outstanding model organism to study this occurrence in insects is the monarch butterfly (*Danaus plexippus*) (Reppert and de Roode, 2018; Reppert et al., 2016). Each fall millions of these colorful butterflies leave their breeding areas in North America and Canada and migrate over more than 4,000 km to their overwintering sites in Central Mexico. But how is a small animal able to find its way and keep a certain direction over such an enormous distance? During navigation and spatial orientation, many insects rely on a variety of cues. Beside local cues such as wind (Dacke et al., 2019; Müller and Wehner, 2007), odors (Buehlmann et al., 2012), or landmarks (Fleischmann et al., 2016; Fleischmann et al., 2018), global cues from the sky provide directional information (for a review see: Wehner, 1984) and are in particular relevant when navigating through unfamiliar areas. Amongst them, the sun is the most dominant orientation reference used by many diurnal arthropods (Byrne et al., 2003; Dacke et al., 2014; el Jundi et al., 2014a; el Jundi et al., 2015a; Giraldo et al., 2018; Lebhardt and Ronacher, 2015; Patel and Cronin, 2020; von Frisch and Lindauer, 1956; Wehner, 1997). Its position can easily be detected as the brightest spot in the sky (Wehner, 1984) and guides monarch butterflies southwards by combining the sun compass signals with time-of-day information in the brain (Merlin et al., 2009; Mouritsen and Frost, 2002). However, when the sun is not visible on cloudy days, polarized skylight can help insects to find their way. When entering the atmosphere, sunlight scattering generates a pattern of polarized light across the celestial dome, which can be used by a variety of day-active insects for orientation (el Jundi et al., 2014a; von Frisch, 1949; Warren et al., 2018; Wehner, 1997; Weir and Dickinson, 2012). Although monarch butterflies possess specialized ommatidia to detect polarized light (Sauman et al., 2005; Stalleicken et al., 2006) it is still unclear if they rely on the polarization pattern of the sky for orientation during their migration. While one study suggests that monarch butterflies use polarized skylight for orientation (Reppert et al., 2004), another study provides evidence that the butterflies cannot use it for migration (Stalleicken et al., 2005). The butterflies seem to rather rely on other cues for orientation, such as the skylight intensity gradient (Stalleicken et al., 2005). Indeed, some insects, such as dung beetles can use skylight intensity information in combination with the polarization pattern to roll their balls along a straight line (el Jundi et al., 2014a). However, another skylight cue is the spectral gradient of the sky which is a consequence of a wavelength-dependent scattering of sunlight (Coemans et al., 1994; el Jundi et al., 2014a). While UV light is distributed uniformly across the sky, green light is scattered stronger towards in the solar hemisphere. This results in a higher ratio of green light/UV light in the solar hemisphere than in the antisolar hemisphere. Some insects, such as bees were found to have an innate prediction of which wavelength refer to which sky hemisphere (Brines and Gould, 1979; Edrich et al., 1979; Rossel and Wehner, 1984) while dung beetles combine these spectral information irrespective of their natural spatial relationship in the sky (el Jundi et al., 2016). Whether monarch butterflies detect the spectral and intensity gradient of the sky is currently unknown.

The aim of this study was to investigate the use of different celestial cues during the southerly migration of the monarch butterflies and to unravel the underlying cue hierarchy. Performing outdoors and indoors flight simulator experiments, we found that the sun is the most important orientation reference for the butterflies but is not sufficient to set the migratory direction. In addition to the sun, we show that the butterflies used polarized light in combination with other cues, such as the skylight intensity gradient, for orientation to maintain a constant migratory direction. When we presented a simulated sun and polarized skylight simultaneously to the butterflies in indoor experiment, the animals maintained constant flight directions in directions that matched the expected southward direction in nature. These results suggest that both the sun and polarization pattern of the sky are used by the butterflies to successfully set the correct direction to reach the overwintering habitats in Central Mexico.

Material and Methods

Experimental animals

Monarch butterflies (*Danaus plexippus*) were caught in October/November 2018/2019 while passing through College Station (TX, USA) during their southward migration. After ensuring that they were not infected by *Ophryocystis elektroscirrha* (Reppert and de Roode, 2018), individual butterflies were transferred in glassine envelopes and housed in an incubator (I-30VL, Percival Scientific, Perry, IA, USA) with a 13:11 h light:dark cycle. During the light phase, the temperature was adjusted to 23°C while it was set to 12°C during the dark phase. The animals were individually fed with 30% honey water every other day.

Flight simulator

We prepared male and female butterflies in the morning prior to each experiment. For this, the scales of the butterflies' dorsal thorax were removed and a tungsten stalk (0.508*152.4 mm, Science Products GmbH, Hofheim, Germany) was dorsally attached to the animals' thorax using an instant contact adhesive glue (multi-purpose impact instant contact adhesive, EVO-STIK, Bostik Ltd, Stafford, UK). After preparation, the animals were kept in envelopes and transferred in a dark box until they were individually tested. To study the butterflies' orientation behavior, we used an indoor and outdoor flight simulator similar to the one described previously (Dreyer et al., 2021, Franzke et al. 2021). At the center of a non-translucent gray barrel, an individual butterfly was connected to an optical encoder (E4T miniature Optical Kit Encoder, US Digital, Vancouver, WA, USA) via its tungsten stalk. The butterfly's heading was recorded with an angular resolution of 3° and a temporal resolution of 200 ms using a data acquisition device (USB4 Encoder Data Acquisition USB Device, US Digital, Vancouver, WA, USA) and a computer with the corresponding software (USB1, USB4: US Digital, Vancouver, WA, USA). At the same time, we filmed the butterfly's performance with a camera (ELP-USBFHD01M-L170, Shenzhen Ailipu Technology Co., Ltd, Nanshan District, Shenzhen, China) from below to ensure that it was constantly flying. All animals that stopped more than once per two minutes of the length of the experiment (i.e., more than two stops when tested four minutes, more than three stops when tested six minutes, or more than four stops when tested eight minutes) were immediately excluded from the study.

Orientation under the natural sky

The Sun Compass

In a first step, we tested the importance of the sun as an orientation reference. To study this, we set up our flight simulator in College Station, TX, USA (30.62° N, -96.36° W) outdoors and tested the heading direction of 19 butterflies under a clear, cloudless sky. The animals were tested during midday, at high sun elevations (>35°) to ensure that they could see the sun while tethered at the center of the flight simulator. After four minutes, we relocated the position of the sun by covering the sun from the butterflies' view by a shading board and simultaneously displacing the sun by 180° to the opposite sky hemisphere using a mirror (size: 20 x 22 cm; experiment: *sun and mirror*; Fig. 1A). We then recorded the butterflies' heading direction over the next two minutes. To next examine whether monarch butterflies interpret a green light spot as the sun, we again recorded the performance of 26 butterflies under the unmanipulated sky at high sun elevation (>35°). Similar to the first experiment (*sun and mirror*), we covered the position of the sun after four minutes. However, this time, we presented a green LED (Emission peak = 520 nm; LZ1-00G102, Osram, San Jose, CA, USA) at an azimuthal distance of 180° to the sun for two minutes. The LED was adjusted to an intensity of ~9.53×10^{13} photons/cm^2/s (measured at the center of the flight simulator) and at an elevation of ~54° (experiment: *sun and sun stimulus*; Fig. 3A). Again, the sun was shaded from the butterflies' view while the LED was turned on.

The polarization compass

To investigate the use of polarized light for migration, we tested the flight performance of 20 butterflies under a linear polarization filter (Fig. 2A). To study this, we mounted the encoder at the center of a Perspex glass sheet (50*50*0.4 cm, Hobbyglas transparent, Gutta Werke GmbH, Schutterwald, Germany). A UV-permeable polarization filter (BVO UV, Bolder Vision Optic, INC., Boulder, CO, USA) with a diameter of 48 cm was mounted on a circular holder on top of the flight simulator, just below the Perspex glass sheet. A small hole at the center of the filter allowed connecting the tethered animal to the encoder. The polarization filter was therefore disconnected from the Perspex sheet/tethered animal and covered the entire dorsal visual field of the flight simulator, allowing only polarized light to enter the flight simulator. By changing the orientation of the polarization filter, we were able to change the angle of polarization, while all other cues remained in place.

All individuals were tested in the evening (sun elevation <30°), to ensure that the animals did not have a direct view to the sun (which was hidden by the non-translucent gray barrel). At the beginning of each experiment, the angle of polarization of the filter was aligned with the main angle of polarization in the sky (i.e., perpendicular to the direction of the sun) and the butterflies' heading directions were recorded for four minutes. Afterwards, the polarizer was turned by 90° for the next two-minute phase (i.e., the angle of polarization of the filter was in line with the sun direction). For the last two minutes, we turned the filter back to its original orientation (perpendicular to the sun direction) and recorded the butterflies' heading direction for another two minutes (experiment: *POL outdoor*; Fig. 2B).

Orientation with respect to single orientation cues

The sun compass

In a second set of experiments, we investigated the use of single orientation cues in an indoor flight simulator. To study the use of a simulated sun as the only orientation reference in indoor experiments, we equipped the flight simulator with two green LEDs (Emission peak = 520 nm) set 180° apart. Both light spots were mounted at an elevation of ~23° and each of them was adjusted to a light intensity of ~1.83×10^{13} photons/cm^2/s (measured at the center of the flight simulator; Fig. 4A). We recorded the orientation of 21 animals with respect to a simulated sun for a total of eight minutes by turning one of the LEDs on. To ensure that the butterflies relied on the displayed cue for orientation, we turned the visual scenery by 180° every two minutes by turning one LED off and simultaneously turning the other one on. We alternated the start position of the stimulus between 0° and 180°. Thus, half of the butterflies experienced the green sun stimulus at 0° first, while the other half experienced the green sun stimulus at 180° first (experiment: *sun stimulus*).

Spectral Contrast

We next studied the orientation behavior of 20 animals with respect to spectral information, similar to how it has been performed in dung beetles (el Jundi et al., 2015b; el Jundi et al., 2016). Therefore, we added two UV LEDs (Emission peak = 365 nm; LZ1-10UV00, Osram, San Jose, CA, USA) next to the green light spots at an elevation of ~23° and adjusted their brightness to the same photon flux as the green lights (~$2.0*10^{13}$ photons/cm^2/s; measured at the center of the flight simulator). At the beginning of each experiment, we turned one green light and the UV light spot on the opposite site on (Fig. 3E). We alternated the start position of the green light spot between 0° (UV light at 180°) and 180° (UV light at 0°). Thus, in half of the animals, the green light spot was at 0°, for the other half of the animals at 180° at the beginning of the experiment. We then recorded the animals' heading over two minutes (minute 1-2). For the next two minutes (minute 3-4), we turned one of the stimuli (either the green or the UV light spot) off. In half of the butterflies, the green light spot was withheld, while in the other half of the animals the UV light spot was turned off. All butterflies experienced both stimuli again for the next subsequent two minutes (minute 5-6) before the other stimulus was turned off (either the green or UV light) for the final two minutes (minute 7-8). Animals that experienced the green light spot during minute 2-4, were presented with a UV light cue during minute 7-8 and *vice versa* (experiment: *spectral contrast*).

The polarization compass

To investigate the use of the polarized light without any additional visual orientation references, we also performed polarization experiments indoors. We attached the Perspex glass sheet and the polarization filter on top of the flight simulator, similar to how we constructed it for the outdoor polarization experiments (Fig. 4F). Because monarch butterflies detect the polarization pattern in the UV range (Sauman et al., 2005; Stalleicken et al., 2006), we mounted six high-power UV LEDs (Emission peak = 365 nm; LZ1-10UV00, Osram, San Jose, CA, USA) on an aluminum plate above the flight simulator. To generate an evenly illuminated polarization stimulus, we added a diffusion filter (216 White Diffusion, LEE Filters, Andover, Hampshire, UK) on top of the Perspex sheet. The intensity of UV light was set to $3.19*10^{14}$ photons/cm^2/s (measured at the center of the flight simulator). At the beginning of each experiment, we aligned the axis of the polarizer either along the 0°-180°-axis or along the 90°-270° axis of the flight simulator. The orientation behavior of 20 butterflies was recorded for a total of eight minutes. Similar to the procedure in the outdoor experiments, the polarization filter remained in place for the first four minutes. The polarization filter was then turned by 90° for the subsequent two minutes, before it was turned back to its original position for the last two minutes of the experiment (experiment: *POL indoor*).

Orientation with respect to the sun and polarization stimulus

In a final experiment, we simulated the polarization pattern and the sun in our indoor flight simulator (Fig. 4H). Therefore, a green light spot was mounted at an elevation of ~23° and adjusted to a light intensity of ~$1.83*10^{13}$ photons/cm^2/s (measured at the center of the flight simulator). We attached the Perspex glass sheet with diffusion filter, the polarization filter and the UV stimulus which was set to $3.19*10^{14}$ photons/cm^2/s (measured at the center of the flight simulator) on top of the flight simulator. To simulate the natural relationship of the cues, the angle of polarization was oriented perpendicular to the green sun stimulus. We let 27 butterflies acclimatize in the flight simulator for two minutes before we recorded their headings for another two minutes (experiment: *sun stimulus and POL*).

Data analysis and Statistics

We imported the flight data into the software MATLAB (Version R2017b, MathWorks, Natick, MA, USA) and analyzed them using custom-written scripts that included the CircStat toolbox (Berens, 2009). The experiments that took eight minutes (*POL* *outdoor*, *sun stimulus*, *spectral contrast*, *POL indoor*) were divided into four flight sections of equal length while experiments performed over six minutes (*sun and mirror*, *sun and sun stimulus*) were divided into three flight sections. The combination experiment (*sun stimulus and POL*) was split into two flight sections of two minutes each. The heading angles of the indoor experiments in which we alternated the position of the stimulus after the first flight section (*spectral contrast*, *POL indoor*), were calculated with respect to the green sun stimulus (0°) or the angle of polarization of the polarizer. For each butterfly, we generated a flight trajectory (Fig. 1B) and calculated the mean heading direction and vector strength within each flight section (over two minutes). As the flight performance of the butterflies can improve over the first minutes of flight (Franzke et al., 2020; Franzke et al. 2021), we focused our analysis on the following three flight sections (minute 3-8). We made an exception for the experiments with the polarization filter (*POL outdoor*, *POL indoor*), where we used the first and fourth flight sections as a control for the change in heading (Fig. 2C&F) and the experiment with the spectral contrast. In the outdoor experiments in which the butterflies viewed the sun during the first four minutes, we excluded all butterflies (*sun and mirror*: 3 out of 19, *sun and sun stimulus*: 7 out of 26) that did not maintain the correct geographical migratory direction (between 110 - 290°, which is the direction to Central Mexico ± 90°) within the first two minute of flight. As we noticed that most of the butterflies tested outdoors under the polarization filter did not keep their migratory direction, we did not filter these data. To analyze whether the animals adjusted their heading direction after manipulation of the visual cues, we calculated the change of heading as the angular difference between two consecutives flight sections. To investigate if the butterflies maintained the correct southward direction with respect to the simulated sun (*sun stimulus*, *sun stimulus and POL*) in our indoor flight simulator, we calculated the butterflies' headings with respect to the astronomical position of the "real" sun at a given time of the day.

The non-parametric Moore's Modified Rayleigh test (Moore, 1980) was used to test for a non-uniform-distribution of the heading directions. Furthermore, we compared the heading directions of different butterfly groups using the Mardia-Watson-Wheeler test. We applied the V-test to test whether the butterflies' change in heading was clustered around 0°/180° or -90°/90° after manipulation of a visual scene. We compared the vector strengths of the butterflies between different experiments using a Kruskal-Wallis test for samples of different groups or the Wilcoxon signed-rank test for samples of the same individuals.

Results

Migratory orientation to the sun

To investigate the importance of different celestial cues on the migration behavior of monarch butterflies, we recorded the flight performance while the animals were tethered at the center of a flight simulator (Fig. 1A). When the butterflies navigated under a fully visible sky, including the sun, they significantly kept a constant heading in the correct south-west direction ($P<0.001$, $R=2.004$; $\mu=221°$; non-parametric Moore's Modified Rayleigh test; $N=16$; Fig. 1B&C, left plot), suggesting that they successfully performed their migratory behavior in our simulator. We then tested the role of the sun for the butterflies' migration. We changed the position of the sun to the opposite sky hemisphere using a mirror while, at the same time, the real sun was covered by a shading board. If the sun is the main orientation reference, the butterflies will follow the sun's relocation by changing their migratory direction accordingly. Indeed, as soon as we displaced the sun, most butterflies (12 out of 16) changed their migratory heading towards a north-east direction (Fig. 1B&C, right plot). Thus, the sun is the main reference that guides the butterflies along their migratory route. However, we found that four butterflies ignored the change in sun position and kept their original bearing (Fig. 1C, right plot), which resulted in a bimodal distribution of heading angles ($P=0.009$, $Z=4.55$; Rayleigh test, $N=16$; Fig. 1C, right plot). We also quantitatively evaluated the butterfly's flight performance by calculating the vector strength – a measure for the flight directedness – prior to and after sun displacement. Interestingly, the butterflies' flight directedness significantly dropped after sun relocation ($P=0.03$, $Z=2.172$, Wilcoxon signed-rank test; Fig. 1D). This result, together with the observation that some butterflies ignored the change in sun azimuth, suggests that the butterflies take additional cues into account to set their migratory direction.

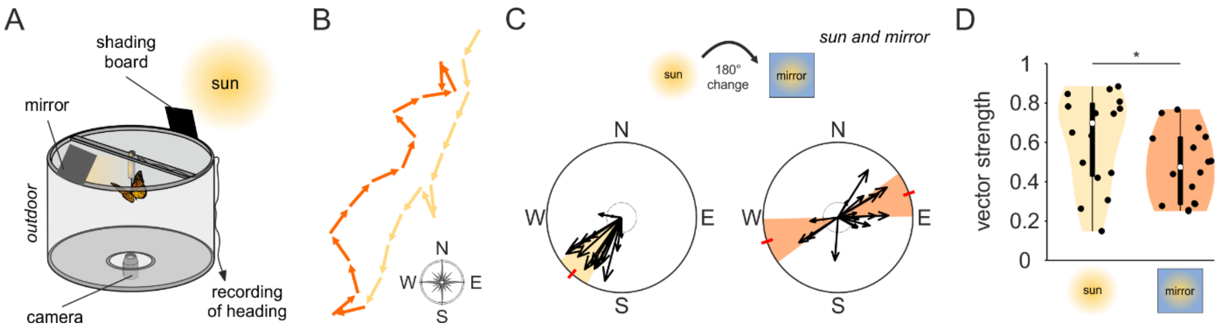

Fig. 1. The position of the sun as important migration reference. (A) Schematic illustration of the experimental setup used to study sun orientation in monarch butterflies. (B) Virtual four-minute flight track of an individual butterfly viewing the sky (yellow arrows). The animal changed its south-west directed flight behavior as soon as the sun was mirrored (orange arrows). (C) Orientation of butterflies (N=16) in a flight simulator that first perceived the natural sky (left plot) and afterwards the mirrored sun (right plot). Each arrow represents the mean heading direction and vector strength *r* of an individual butterfly. The strength of the vectors can vary between zero (disoriented) and one (perfectly oriented). The inner dashed circle indicates a vector strength of 0.2 and the perimeter of the plot a vector strength of 1. The yellow and orange sectors show the 95% quantile of the animals' significant mean direction indicated by the red line. (D) The animals' vector strength significantly decreased after the relocation of the sun's position (P=0.03, Z=2.172, Wilcoxon signed-rank test). White dots indicate the median vector strength. The black boxes show the interquartile range and thin black lines extend to the 1.5 x interquartile range. Black dots show the individual data points and shaded area represents their density. * indicates a significant difference of $p < 0.05$.

The relevance of polarized light for migration

We next wondered if the butterflies use the polarized skylight as migratory cue by recording the butterflies' headings under a polarization filter at low sun elevations (Fig. 2A). Interestingly, the animals did not maintain their southerly migratory direction under the polarizer but rather showed arbitrary headings (P=0.276, R=0.655; non-parametric Moore's Modified Rayleigh test; N=20; Fig. 2B first plot). This suggests that under these conditions, the butterflies were not able to set the migratory direction. When the angle of polarized light remained in place (0° change) most of the animals (15 out of 20) did not alter their course by more than 45° (Fig. 2C', 2C'', 2C'''). Two individuals performed a 180° turn, a behavior that would be expected if the butterflies used polarized light as the main orientation reference. In general, the butterflies were able to keep steady flight directions under the polarization filter (P<0.001, v=12.861, axial V-test, expectation: 0°; Fig. 2C’). When the polarizer was turned by 90°, the butterflies flight direction was affected, which resulted in a group orientation towards the North-East (P=0.027, R=1.096; μ=54°; non-parametric Moore's Modified Rayleigh test; N=20; Fig. 2B third plot). Interestingly, we found that many butterflies followed the rotation of the polarization filter (Fig. 2D’’’). In general, significantly more animals changed their flight course after a rotation of the polarizer than when the angle of polarization was unaltered (*no turn* vs. *POL turn 1*: P=0.038, χ^2=4.286; Chi-square test; *no turn* vs. *POL turn 2*: P=0.008, χ^2=7.033; Chi-square test; N=20; Fig. 2G), indicating the use of polarized light for orientation. However, not all butterflies followed the 90°-turn of the polarizer (*POL turn 1*: P=0.757, v=-2.467, axial V-test, expectation: 90°). Many animals changed their flight course by an arbitrary azimuthal direction, which resulted in a random change in heading (P=0.467, Z=0.773, axial Rayleigh test; Fig. 2D’). To ensure that these results were not affected by the flight performance of individual animals, we also correlated the butterflies’ change in heading with the corresponding vector strength (Fig. 2C’’, 2D’’). While the butterflies with a high directness showed low changes in heading when the polarizer remained in place (Fig. 2C’’), many well-directed butterflies changed their heading after the 90°-turn of the polarizer (Fig. 2D’’).

When the polarization filter was turned by 90°, we likely have induced a cue conflict situation that led to the arbitrary changes in heading direction of the butterflies. We therefore wondered how the butterflies’ heading choices are affected when the polarizer is turned back to its original orientation and, thus, we eliminate the cue conflict situation (Fig. 2E). Again, many butterflies changed their heading direction by about 90° (Fig. 2G) while others changed their heading by a random azimuthal angle/kept their direction as a response of the second polarizer turn (Fig. 2E’&E’’). Interestingly, the change in heading was significantly clustered along the 0° or 180° axis when we compared the butterflies’ heading direction prior to the first polarizer turn with the ones taken after the second polarizer turn (P<0.001, v=-27.730, axial V-test, expectation: 0°; Fig. 2F’&F’’). Thus, the butterflies returned to their original heading direction after the polarizer was turned to its original position. Taken together, these results suggest that polarized light is used in combination with another cue, such as the celestial intensity and/or spectral gradient, for orientation when the sun is hidden from the butterfly's view.

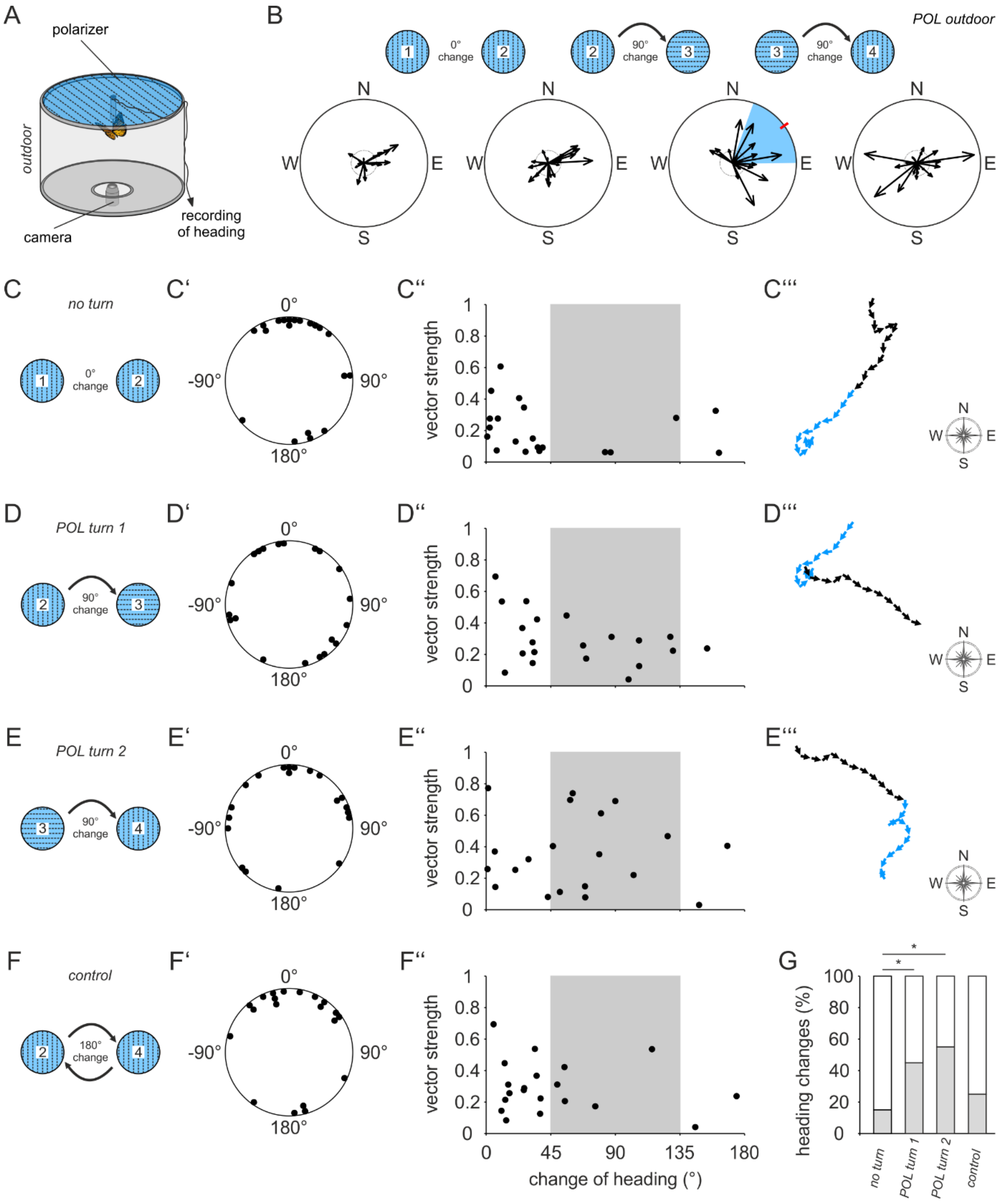

Fig. 2. Orientation with respect to polarized light. (A) Schematic illustration of the experimental setup used to study polarization orientation in monarch butterflies. (B) Orientation of butterflies (N=20) in a flight simulator with the angle of polarized light aligned (first, second,

and fourth plot) or perpendicular to the main polarization angle in the sky (third plot). Each arrow represents one butterfly. The mean direction of the animals is shown as a red line while the blue sector indicates the 95% quantile. The inner dashed circle indicates a vector strength of 0.2 and the perimeter of the plot a vector strength of 1. (C-F) Change of heading (bin size: 5°) when the polarizer orientation remained the same (C, *no turn*, $P<0.001$, $v=12.861$, axial V-test, expectation: 0°, first and second plot in B), when the polarizer was turned by 90° in between (D, *POL turn 1*, $P=0.757$, $v=-2.467$, axial V-test, expectation: 90°; second and third plot in B), and when the polarizer was turned by 90° for a second time (D, *POL turn 2*, $P=0.881$, $v=-5.277$, axial V-test, expectation: 90°; third and fourth plot in B). We also compared the changing in heading between the direction taken prior to and after the second polarizer turn (F, *control*, $P<0.001$, $v=-27.730$, axial V-test, expectation: 0°, second and fourth plot in B). Each circle in C' - F' represents the angular difference in heading of one butterfly. C''- F'' show the same change of heading in relation to the vector strength in the phase before turning. The gray boxes indicate a 90° ± 45° change of heading. C'''- E''' shows the virtual flight track of one butterfly before (black arrows in C'''&E''', blue arrows in D''') and after (blue arrows in C'''&E''', black arrows in D''') turning the polarization filter. (G) Significant more animals changed their heading by 90° ± 45° (gray) when the polarizer was turned (*POL turn 1*, *POL turn 2*) than when its orientation remained the same (*no turn*, *control*; *no turn* vs. *POL turn 1*: $P=0.038$, $\chi^2=4.286$; Chi-square test; *no turn* vs. *POL turn 2*: $P=0.008$, $\chi^2=7.033$; Chi-square test; N=20; Fig. 2G).

The use of a simulated sun as orientation reference

To understand how celestial cues are combined in the monarch butterfly compass, we next aimed to study the migratory behavior under well-controlled condition in indoor flight simulators. However, we first wondered if the butterflies treat a green light stimulus as the sun, similar to what has been found in dung beetles (el Jundi et al., 2015a). For this, we recorded the butterflies' headings under the natural sky first. Similar to the experiments with the mirrored sun, we subsequently shaded the position of the sun and introduced a green light spot at 180° to the real sun (Fig. 3A). The butterflies kept their expected migratory southward direction when they had the sun for orientation ($P<0.001$, $R=2.043$; $\mu=186°$; non-parametric Moore's Modified Rayleigh test; N=19; Fig. 3B left plot). When the sun was blocked and the green light spot was presented, the butterflies changed their flight course significantly by 180° ($P<0.001$, $u=3.882$, V-test, expectation: 180°; Fig. 3C&D) and headed northwards ($P<0.001$, R=1.649; μ=3°; non-parametric Moore's Modified Rayleigh test; N=19; Fig. 3B right plot). This shows that the butterflies interpret the green light spot as the sun, which allows us to effectively simulate the sun as a green light spot in our indoor experiments.

The use of spectral vs. intensity cues

Our experiments under the polarization filter suggest that the butterflies use the pattern of polarized light in combination with another cue. In addition to polarized light, sunlight scattering leads to a spectral gradient in the sky. This gradient is characterized by a high amount of green light towards the sun and a relatively high UV light in the opposite hemisphere to the sun (Coemans et al., 1994; el Jundi et al., 2014b). To investigate if the butterflies can use the spectral contrast for orientation, we mounted a UV light spot 180° opposite to the green sun stimulus in our flight simulator (Fig. 3E). We then tested the butterflies' ability to use the spectral information for orientation by turning either of these cues off (Fig. 3F upper panel), similar to how it has been tested in dung beetles (el Jundi et al., 2015b; el Jundi et al., 2016). When both cues were presented at the beginning, the butterflies kept constant heading direction with a high vector strength towards the hemisphere of the UV light cue (P<0.001, R=1.906; μ=40° with respect to the UV LED; non-parametric Moore's Modified Rayleigh test; N=20; Fig. 3F upper panel: first plot). When we then withhold the green sun stimulus (i.e., the butterflies had the UV light cue as the only orientation reference), the animals maintained their headings towards the UV stimulus (P<0.001, R=1.739; μ=10° with respect to the UV LED; non-parametric Moore's Modified Rayleigh test; Fig. 3F, upper panel: second plot). As a result, the change in heading was clustered around 0° (P=0.002, u=2.796, V-test, expectation: 0°; Fig. 3F lower panel: first plot &3G). We then added the green sun stimulus to the scene and allowed the butterflies to orient with respect to both cues, before we then turned off the UV light spot (Fig. 3F, upper panel: third and fourth plot). If the butterflies could use spectral (rather than brightness) information for orientation, we expect them to maintain their flight direction even when the UV cue was not available. However, the majority of butterflies changed their heading direction by 180° in the absence of the UV light (P=0.03, u=1.88, V-test, expectation: 180°; Fig. 3F lower panel: second plot &3G). This suggests that the butterflies do not use the spectral, but rather the intensity, gradient of the sky as an orientation reference during their migration.

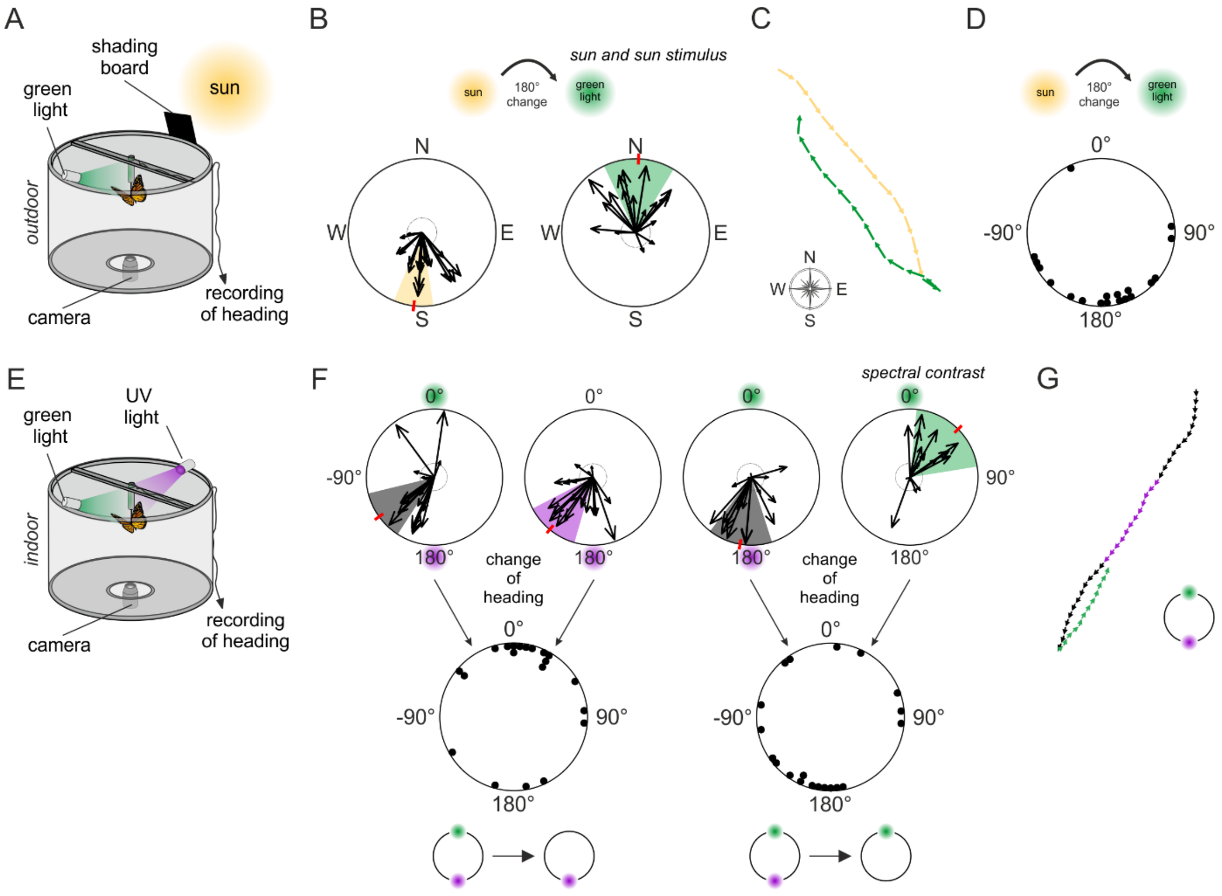

Fig. 3. Orientation with respect to a spectral contrast. (A) Schematic illustration of the experimental setup used to study the use of a green light spot as simulates sun in monarch butterflies. (B) Orientation of butterflies (N=19) first perceiving the natural sky (first plot) and after replacing the sun by the green light spot set 180° apart (second plot). Each arrow represents one butterfly. The mean direction of the animals is shown as a red line while the colored sector indicates the 95% quantile. (C) Virtual four-minute flight track of one butterfly viewing the sky (yellow arrows). The animal changed its' south-east directed flight behavior as soon as the position of the sun was shaded, and the simulated sun was displayed (green arrows). (D) The change of heading (bin size: 5°) when the sun was replaced by a green light spot (P<0.001, u=3.882, V-test, expectation: 180°). Each circle represents the angular difference in heading of one butterfly. (E) Schematic illustration of the experimental setup used to study the butterflies' orientation with respect to a spectral contrast. (F) Upper panel: orientation of butterflies (N=20) perceiving a spectral contrast with green and UV light (first and

third plot) or only UV light (second plot) / green light (fourth plot) alone. Plot conventions as in B. Lower panel: the change of heading (bin size: 5°) when the green (left plot) or UV light (right plot) was turned off. Plot conventions as in D. (G) Virtual eight-minute flight track of one butterfly with respect to a spectral contrast (black arrows), a UV (purple arrows) and a green light spot (green arrows).

Single celestial cues for orientation

Our data show that the sun and the polarized light (plus intensity information of the sky) are prominent cues that the butterflies rely on during their migration. We next wondered, if one of these cues is sufficient to guide the butterflies on their southward migration. We therefore presented the simulated sun to the butterflies first in an indoor flight simulator (Fig. 4A). The butterflies showed arbitrary headings when the green sun stimulus was the only source of reference (P=0.374, R = 0.572; non-parametric Moore's Modified Rayleigh test; N=21; Fig. 4B). However, the butterflies were by no means disoriented. They were able to keep highly directed flight directions (Fig. 4B, C&E) and responded to a 180° relocation of the sun stimulus with a consistent change in their heading direction (P=0.001, u=2.957, V-test, expectation: 180°; Fig. 4C&D). Thus, the butterflies used the simulated sun for orientation but not to maintain their migratory direction. Interestingly, the vector strength was lower when the butterflies navigated with the simulated sun indoors (P=0.015, χ^2=5.88, Kruskal-Wallis test) compared to the performance of the butterflies to the real sun outdoors (Fig. 4E). This was not the case, when we presented the simulated sun outdoors (P=0.658, Z=0.443, Wilcoxon signed-rank test), which suggests that the sun on its own does not seem to be sufficient to elicit migratory heading directions in monarch butterflies.

We then tested the butterflies under the polarization filter indoors (Fig. 4F). With the polarized light as the only orientation reference, only a few animals were able to keep constants headings for a short flight sequence while most of the butterflies rotated around their body for the entire experiment, suggesting that they were disoriented (Fig. 4G). Not surprising, the butterflies did not respond to a polarizer turn. These results are well in line with our observation in nature (Fig. 2) and further confirm that polarized light is used in combination with other celestial cues, such as the sun or the skylight intensity gradient, for orientation.

Sun and polarized light induce migratory headings

As none of the presented celestial cues was sufficient for the butterflies to set their migratory flight direction, we next presented the simulated sun in combination with the simulated polarized skylight to the animals (Fig. 4H). Under this scenario, the butterflies performed well-oriented flights with a high directedness, similar to what we observed outdoors. Moreover, the butterflies maintained flight headings towards the hemisphere of the green sun stimulus ($P<0.001$, $R=1.979$; $\mu=284°$; non-parametric Moore's Modified Rayleigh test; Fig. 4I). To test, if these directions were in line with the actual migratory direction in nature, we calculated the headings with respect to the geographical position of the real sun at the time/date of the experiments. In other words, we treated the flight directions of the butterflies as if they were performing their flights outdoors under the natural sky. Interestingly, we found that the butterflies significantly kept a south-west direction in our indoor experiments ($P<0.001$, $R=1.751$; $\mu=215°$; non-parametric Moore's Modified Rayleigh test; Fig. 4J). Although the variance of the heading directions was significantly higher in the indoor experiment ($P=0.009$, $W=9.492$, Mardia-Watson-Wheeler test), this direction matched very well with the migratory direction observed in nature under a clear sky (Fig. 4K; same data as in Fig. 1C first plot). This suggests that the butterfly’s compass requires at least the sun and polarized light to induce the southward migration in monarch butterflies.

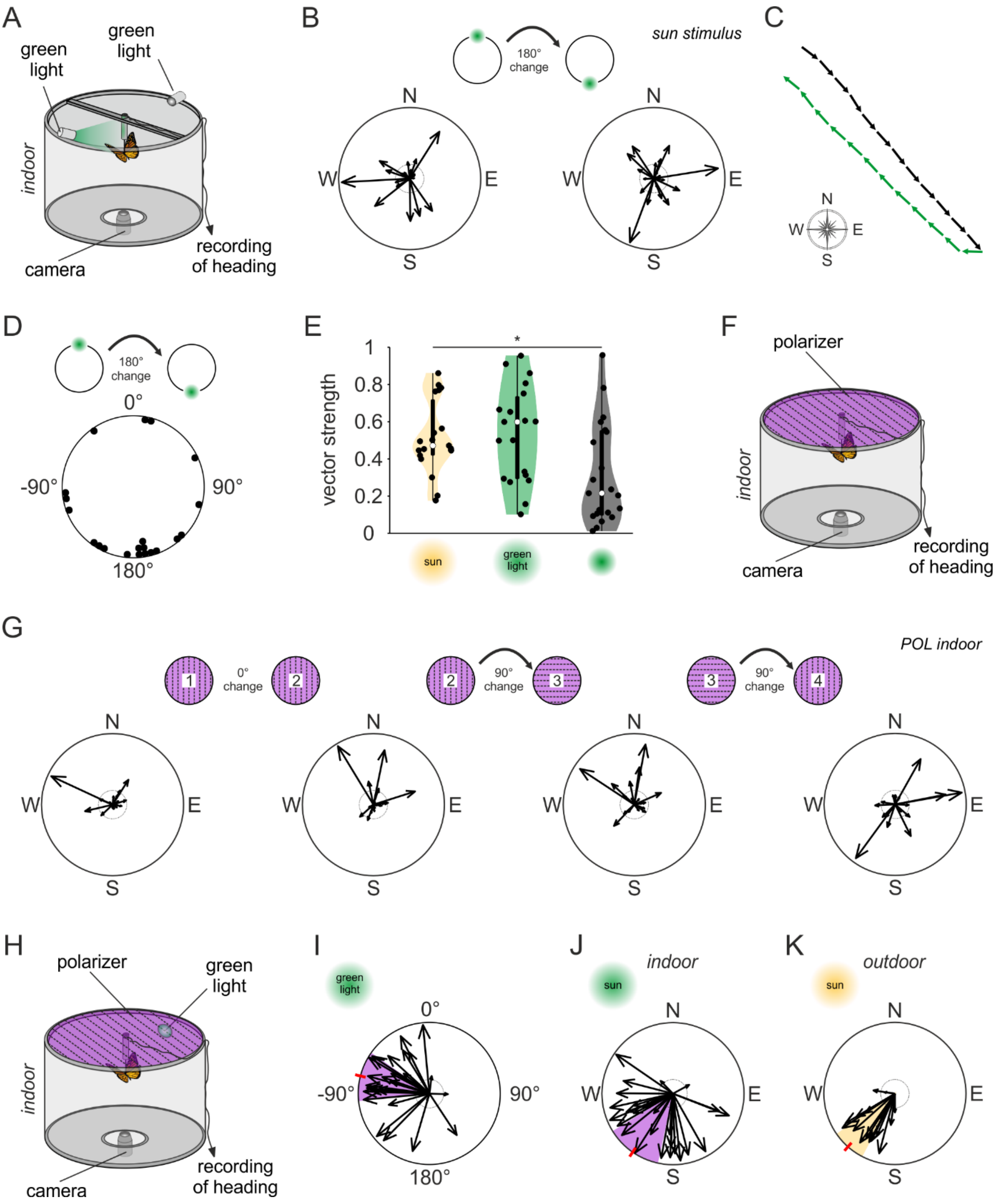

Fig. 4. Orientation with respect to the sun and polarization stimulus. (A) Schematic illustration of the experimental setup used to study the butterflies' orientation with respect to a simulated sun. (B) Orientation of butterflies (N=21) before (left plot) and after a relocation of the sun stimulus by 180° (right plot). Each arrow represents one butterfly. (C) Virtual four-minute flight track of one butterfly with respect to a simulated sun before (black arrows) and after a 180° relocation (green arrows). (D) The change of heading (bin size: 5°) after a 180°

relocation of the simulated sun (P=0.001, u=2.957, V-test, expectation: 180°). Each circle represents the angular difference in heading of one butterfly. (E) The animals' vector strength was about the same level when flying under the natural sky viewing either the real or a simulated sun (P=0.658, Z=0.443, Wilcoxon signed-rank test). Animals tested indoors with respect to a simulated sun performed significantly worse than butterflies perceiving the actual sun (P=0.015, χ^2=5.88, Kruskal-Wallis test). White dots indicate the median vector strength. The black boxes show the interquartile range and thin black lines extend to the 1.5 x interquartile range. Black dots show the individual data points and shaded area represents their density. * indicates a significant difference of $p < 0.05$. (F) Schematic illustration of the experimental setup used to study polarization orientation in the butterflies. (G) Orientation of butterflies (N=20) with the angle of polarized light aligned to the North-South axis (first, second, and fourth plot) or perpendicular to it (third plot). Plot conventions as in B. (H) Schematic illustration of the experimental setup used to study the butterflies' orientation with respect to a combination of polarized light and a simulated sun. (I-K) Orientation of butterflies with respect to a simulated sun and linear polarized light (N=27, I&J) or the sky (K, same data as in Fig. 1C first plot). I shows the actual headings of the butterflies tested indoors and J the headings after we shifted them in such a way the position of the LED matched the portion of the 'real' sun. Plot conventions as in B. The mean direction of the animals is shown as a red line while the colored sector indicates the 95% quantile.

Discussion

Here, we tested the use of different celestial cues in monarch butterflies during their annual fall migration. In a series of experiments, we showed that, although the sun is the most important orientation reference, additional skylight cues are required to set the migratory direction in monarch butterflies. We therefore tested if the butterflies use polarized light for orientation and found evidence for a polarization compass in the butterflies. In indoor experiment, we further found that the animals rely on an intensity rather than spectral information for orientation. In our experiments, polarized light was used to maintain a directed flight course when it was combined with celestial gradients. However, the butterflies were not able to set the migratory direction under this condition. When we presented a combination of polarized light and the position of the sun in indoor experiments, the butterflies kept a migratory south-west direction.

Celestial cues and their hierarchy in monarch butterflies

The sun compass

A number of previous studies showed the importance of the sun as an orientation cue during the migration of the monarch butterflies (Froy et al., 2003; Heinze and Reppert, 2011; Mouritsen and Frost, 2002; Reppert, 2006). By mirroring the sun, we here confirmed that the sun is clearly the most important migratory reference for most monarch butterflies, similar to what has been shown in other arthropods including dung beetles (Byrne et al., 2003; Dacke et al., 2014; el Jundi et al., 2014a; el Jundi et al., 2015a) and mantis shrimps (Patel and Cronin, 2020). Interestingly, not all tested butterflies ranked the sun highest in their compass system and ignored the change in sun position in our experiments, which can also be observed in mantis shrimps (Patel and Cronin, 2020). This shows that even the single-minded migration of the butterflies underlies an interindividual variability in how animals weight different orientation cues, an observation that has previously been reported for the orientation behavior of non-migratory monarch butterflies (Franzke et al., 2020). As in the behavioral study by Franzke et al. (2020), the sun was simulated by a green light spot in previous indoor experiments as well as during electrophysiological recordings in monarch butterflies (Franzke et al. 2022; Beetz et al., 2021; Heinze and Reppert, 2011; Nguyen et al., 2021). We now demonstrated for the first time that the butterflies interpret a bright green light stimulus as the “real” sun. This is in line with the way other insects treat a green light stimulus, such as bees (Brines and Gould, 1979; Edrich et al., 1979; Rossel and Wehner, 1984) as well as dung beetles (el Jundi et al., 2015a). However, when we tested the butterflies indoors with the simulated sun being the only orientation reference, the animals did not head into the correct migratory direction but rather showed arbitrary headings (Fig. 3A-D). This is different to dung beetles, which maintain the same angle when tested indoors with an artificial sun and outdoors with respect to the “real” sun (el Jundi et al., 2015a). One possible explanation is the different behavioral context of these animals. Dung beetles use the position of the sun for straight line orientation to disperse (arbitrary headings) from a food source in a random direction (Baird et al., 2010; Byrne et al., 2003; Dacke et al., 2021) relying on a short-term memory (el Jundi et al., 2016). In contrast to this, the specific geographical direction migrating monarch butterflies have to set must be genetically determined and maintained by the use of a time-compensated sun compass (Froy et al., 2003; Merlin et al., 2009; Mouritsen and Frost, 2002). It is likely that for using such a compass multiple cues need to be integrated and butterflies switch to a dispersal when only perceiving one of them. Taken together, due to the lack of additional skylight cues in our indoor simulators, the butterflies do not exhibit their migratory behavior but switch to a dispersal behavior (arbitrary headings) similar to the behavior exhibited by non-migrating monarch butterflies (Franzke et al. 2021).

The polarization compass

On overcast days, when the position of the sun is not visible, many arthropods can use polarized light for orientation (el Jundi et al., 2014a; Patel and Cronin, 2020; von Frisch, 1949; Wehner, 1997). When we presented polarized light and excluded the position of the sun, we found that monarch butterflies were only able to maintain a course when tested outdoors. However, the animals did not display a migratory group orientation but arbitrary headings which is in line with a previous study using a similar experimental approach (Stalleicken et al., 2005). In the same study, a 90° turn of the polarizer led to arbitrary changes of the butterflies' flight directions suggesting that the butterflies do not use polarized light for orientation (Stalleicken et al., 2005) while in another study the animals precisely followed the angle of polarization (Reppert et al., 2004). Our findings support the results by Stalleicken et al. (2005) as we did not observe a significant change of heading by ±90° on a group level. However, the butterflies in our experiment more often turned by ±90° when the polarizer was turned than in the control situation. This suggests that the polarized light is used by some individuals, again showing the interindividual variability, and that the pattern of polarized light is ranked lower than the sun. This is similar to diurnal dung beetles (el Jundi et al., 2014a) and mantis shrimps (Patel and Cronin, 2020) but contradicts findings in ants which weight the sun and polarized equally (Lebhardt and Ronacher, 2014). When we turned the polarization filter by 90°, we generated a conflict between the direction of the angle of polarization and other celestial cues such as intensity and spectral gradients. Such a conflict of cues can cause a loss of orientation (Dreyer et al., 2018) or induce an insect to head into a new intermediate direction, as it has been shown for desert ants (Wehner et al., 2016). This is also in line with our findings that the butterflies chose the same headings when the angle of polarization was turned back to its original orientation and thereby aligned in the correct position with the remining celestial cues. However, additional control experiments are necessary to exclude the possibility that the change in intensity introduced by the polarization filter turn, rather than the conflict in orientation cues affected the orientation behavior of the butterflies. Together with our finding that only few animals performed well-oriented flights when only the polarized light was available indoor, it is likely that monarch butterflies combine the information of skylight polarization with other cues such as celestial gradients and especially the position of the sun for orientation. This is similar to dung beetles which were found to combine skylight polarization with the intensity contrast of the sky for orientation (el Jundi et al., 2014a).

Spectral Contrast

In contrast to dung beetles (el Jundi et al., 2015b) and bees (Brines and Gould, 1979; Edrich et al., 1979; Rossel and Wehner, 1984), we did not find any evidence for the use of spectral information as orientation reference in monarch butterflies. The butterflies rather relied on intensity information which is also reflected by the animals' neuronal responses to different monochromatic light cues (Heinze and Reppert, 2011; Nguyen et al., 2021) and shown in previous behavioral studies where the butterflies did not distinguish between different colors of stimuli (Franzke et al., 2020; Franzke et al. 2021). Taken together, monarch butterflies primarily rely on information of the sun's position and switch to polarized light cues when the sun's view is blocked. They hereby seem to combine the angle of polarization with the intensity information provided by the sky to keep a constant heading. Although the butterflies use these different celestial cues to maintain a directed course, they were only able to set the migratory direction in indoor experiments when combining the pattern of polarized light with the position of the sun.

Combination of celestial cues

Interestingly, the butterflies headed in arbitrary directions when the sun was their only orientation reference and in contrast to other insects (Dacke et al., 2014; von Frisch, 1949; Wehner, 1997; Weir and Dickinson, 2012) they were not able to use the angle of polarization for orientation when not combined with other cues. This clearly demonstrates that single cues are not sufficient, and a combination of cues is required to set the correct migratory direction in monarch butterflies. During the fall migration, these butterflies use a time-compensated sun compass (Froy et al., 2003; Merlin et al., 2009; Mouritsen and Frost, 2002) which demands the integration of multiple information. Our indoor experiments suggests that this compass is only operating when the butterflies view a combination of the sun's position and polarized light. This raises the question of how the butterflies combine these cues. While one option is that they have a matched filter (Wehner, 1987) for the natural relationship between celestial cues like ants and bees (Brines and Gould, 1979; Edrich et al., 1979; Lebhardt and Ronacher, 2015; Rossel and Wehner, 1984; Wehner, 1997), such a matched filter was found to be not required for dispersal behavior after taking celestial snapshots (el Jundi et al., 2015b; el Jundi et al., 2016). Our outdoor experiments provide evidence that a conflict of celestial cues causes a change in the animals' orientation performance. Thus, it is likely that the butterflies own a matched filter for skylight information. However, this needs to be investigated in more detail in future experiments presenting a simulated sun and the angle of polarization indoors but this time setting both cues in conflict.

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4 Manuscript II:

Spatial orientation based on multiple visual cues in non-migratory monarch butterflies

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RESEARCH ARTICLE

Spatial orientation based on multiple visual cues in non-migratory monarch butterflies

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ABSTRACT

Monarch butterflies (*Danaus plexippus*) are prominent for their annual long-distance migration from North America to their overwintering area in Central Mexico. To find their way on this long journey, they use a sun compass as their main orientation reference but will also adjust their migratory direction with respect to mountain ranges. This indicates that the migratory butterflies also attend to the panorama to guide their travels. Although the compass has been studied in detail in migrating butterflies, little is known about the orientation abilities of non-migrating butterflies. Here, we investigated whether non-migrating butterflies – which stay in a more restricted area to feed and breed – also use a similar compass system to guide their flights. Performing behavioral experiments on tethered flying butterflies in an indoor LED flight simulator, we found that the monarchs fly along straight tracks with respect to a simulated sun. When a panoramic skyline was presented as the only orientation cue, the butterflies maintained their flight direction only during short sequences, suggesting that they potentially use it for flight stabilization. We further found that when we presented the two cues together, the butterflies incorporate both cues in their compass. Taken together, we show here that non-migrating monarch butterflies can combine multiple visual cues for robust orientation, an ability that may also aid them during their migration.

KEY WORDS: Navigation, Insect, Vision, Sun compass, Landmarks, Lepidoptera

INTRODUCTION

Despite their tiny brains, insects exhibit incredible orientation behaviors that range from simple compass orientation (Byrne et al., 2003; el Jundi et al., 2019) to more complex behaviors such as path integration (Collett and Collett, 2000; Heinze et al., 2018) or long-distance migration (Dreyer et al., 2018b; Merlin and Liedvogel, 2019; Warrant et al., 2016). One prominent model organism for the study of spatial orientation in the context of migration is the monarch butterfly (*Danaus plexippus*) (Reppert and de Roode, 2018; Reppert et al., 2016). These colorful butterflies migrate every year over more than 4000 km from North America and Canada to their overwintering habitat in Central Mexico. To find their route, they rely on celestial compass cues, such as the sun and polarized light (Mouritsen and Frost, 2002; Froy et al., 2003; Reppert et al., 2004; Reppert, 2006; Heinze and Reppert, 2011), and the Earth's magnetic field (Guerra et al., 2014), with the sun being their main orientation reference during migration (Stalleicken et al., 2005). In order to do this, they compensate their sun compass based on time-of-day information from circadian clocks in the brain (Sauman et al., 2005) and/or the antennae (Merlin et al., 2009, 2011; Guerra et al., 2012) to keep a constant southerly migratory direction over the entire course of a day (Mouritsen and Frost, 2002; Froy et al., 2003). Besides these cues, observations of heading directions in freely migrating butterflies indicate that they additionally rely on terrestrial cues and adjust their migratory direction from south–southwest to south–southeast as soon as they reach the mountains of the Sierra Madre Oriental (Calvert, 2001). Although it is still unclear whether the monarch butterflies use terrestrial cues in combination with skylight cues, it is known that migrating Bogong moths constantly integrate visual landmarks with the Earth's magnetic field to maintain a directed course (Dreyer et al., 2018a).

To obtain a robust orientation compass, it is well established that many insects use a combination of visual cues from their environment. Ants combine skylight (Lebhardt and Ronacher, 2015; Wehner, 1997) and terrestrial cues, such as the panoramic skyline (Judd and Collett, 1998; Collett and Collett, 2002; Durier et al., 2003; Graham and Cheng, 2009a), to define the desired homeward direction. Integration of multiple visual cues is a common strategy in insects, allowing them to keep track of their heading direction irrespective of their behavioral state. Neurobiological studies in flying fruit flies showed that the insect's internal compass encodes the entire visual scene in a highly flexible manner (Fisher et al., 2019; Kim et al., 2019). This dynamic coding of visual cues allows an insect to constantly integrate multiple cues, such as a panoramic scenery, in its compass and to set it in relation to the sun's position. A very similar internal compass network not only steers migration to Mexico in monarch butterflies (Heinze and Reppert, 2011; Heinze et al., 2013), but also likely guides animals through their environment in their non-migrating phase.

The present study is a first step in investigating how non-migrating monarch butterflies use single visual cues (simulated sun and panoramic skyline) and a combination of these cues for orientation. We presented the cues to the butterflies while the animals were tethered at the center of an LED flight simulator. Although these butterflies were not in their migratory state, we found that they were able to keep a constant heading direction for the entire flight sequence with respect to a simulated sun. When we presented a panoramic skyline to the butterflies, they were also able to keep constant headings with respect to this stimulus, but only did so for short flight periods. Thus, most butterflies seem to use the panoramic skyline for flight stabilization. When the simulated sun and the panoramic skyline were presented together, we found that

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the butterflies used both cues for orientation. Their directedness dropped if they had only one cue for orientation. Our results show that, irrespective of their migratory or internal state, monarch butterflies can maintain a directed heading based on a simulated sun and terrestrial cues. These findings will allow us to investigate the behavioral and neural mechanisms of how these animals maintain a directed course not only in migrating but also in non-migrating butterflies.

MATERIALS AND METHODS

Experimental animals

Monarch butterfly [*Danaus plexippus* (Linnaeus 1758)] pupae were obtained from the butterfly supplier Costa Rica Entomology Supply (butterflyfarm.co.cr). The pupae were reared in an incubator (HPP 110 and HPP 749, Memmert GmbH+Co. KG, Schwabach, Germany) at 25°C and 80% relative humidity under a 12 h:12 h light:dark cycle. After eclosion, the adult butterflies were kept inside a flight cage in an incubator (I-30VL, Percival Scientific, Perry, IA, USA) under a 12 h:12 h light:dark cycle. The incubator was set to 25°C in the light phase and 23°C in the dark phase and to a constant relative humidity of 50%. The butterflies had free access to a feeder containing 15% sucrose solution. In our experiment, we used adult butterflies of both sexes 3–12 days after eclosion. For all experiments, we tested a new group of butterflies.

Prior to the experiments, each butterfly's thorax was cleared of scales, and a tungsten stalk (0.508×152.4 mm, Science Products GmbH, Hofheim, Germany) was attached to the thorax dorsally using an instant adhesive glue (multi-purpose impact instant contact adhesive, EVO-STIK, Bostik Ltd, Stafford, UK). Before the butterflies were tethered in the flight simulator, they were kept in a clear plastic container with access to 15% sucrose solution for at least 3 h in darkness to allow the glue to harden.

Flight simulator

All experiments were performed indoors in an LED flight simulator (Fig. 1A). Similar to previous studies (Mouritsen and Frost, 2002; Dreyer et al., 2018a,b), the heading directions of individual butterflies were recorded by connecting the tungsten wire to an optical encoder (E4T miniature Optical Kit Encoder, US Digital, Vancouver, WA, USA) at the center of the flight simulator. Body orientations were recorded at a temporal resolution of 200 ms and an angular resolution of 3 deg, and sent via a digitizer (USB4 Encoder Data Acquisition USB Device, US Digital) to a computer with the corresponding software (USB1, USB4: US Digital). To present different visual stimuli to the butterflies, the inner surface of the arena was equipped with an array of 2048 RGB LEDs (16×16 APA102C LED Matrix, iPixel LED Light Co., Ltd, Baoan Shenzhen, China). The color and intensity of all LEDs were controlled by a Raspberry Pi (Raspberry Pi 3 Model B, Raspberry Pi Foundation, UK) and a custom written Python script.

The sun compass in butterflies

To simulate the sun, one LED, at an elevation between 5 and 10 deg, was set to green light (emission peak at approximately 516 nm; intensity of ∼5.2×10^{12} photons cm^{-2} s^{-1}, measured at the center of the arena) while the remaining LEDs of the arena were set to blue light (emission peak at approximately 458 nm; intensity of ∼4.61×10^{10} photons cm^{-2} s^{-1} per LED, measured at the center of the arena; experiment: green sun). Individual butterflies were tethered at the center of the arena and their headings were recorded for 8 min (Fig. 2B). The position of the stimulus was switched by 180 deg every 2 min, to ensure that the animals relied on the stimulus presented for orientation. The start position of the sun stimulus was pseudorandomized. Thus, half of the butterflies experienced the sun stimulus at 0 deg first (0/180/0/180 deg), while for the other half of the butterflies the sun stimulus was set at 180 deg first (180/0/180/0 deg).

To understand which features, the spectral or intensity information, of the sun stimulus butterflies used, we performed an additional experiment (again over 8 min; Fig. 2D) in which we excluded the spectral information from the sun stimulus. Thus, the animals' behavior was tested by performing the same experiment as with the green sun but with a blue LED that had the same spectrum as the remaining blue LEDs of the arena but was much brighter (∼5.2×10^{12} photons cm^{-2} s^{-1}, measured at the center of the arena; experiment: blue sun).

To exclude the possibility that butterflies used any additional cues in the experimental setup or room, a negative control experiment was performed for 8 min with all LEDs set to the same blue wavelength and intensity (experiment: no cue; Fig. 2C).

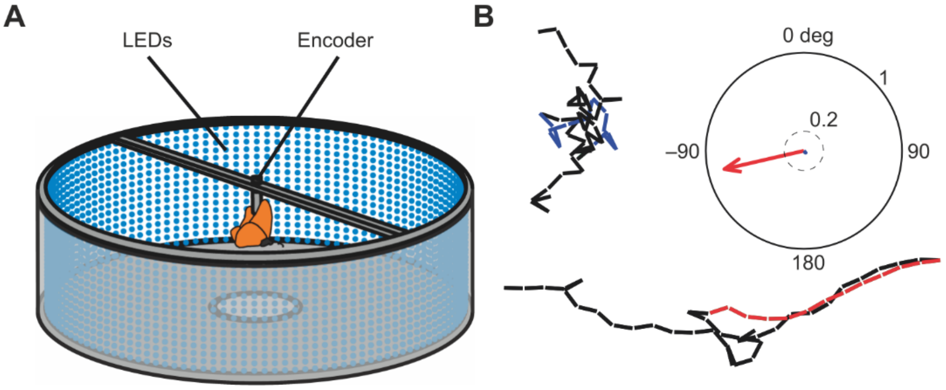

Fig. 1. The orientation of tethered monarch butterflies in an LED flight simulator. (A) Schematic illustration of a monarch butterfly tethered at the center of the LED flight simulator. The inner surface of the arena is equipped with 2048 RGB LEDs. While presenting visual stimuli to the butterflies, their heading directions were monitored using an optical encoder. (B) Virtual 8-min flight tracks of a disoriented (upper, left trajectory) and a well-oriented (lower trajectory) butterfly (bin size: 10 s). The red and blue portion of the trajectories indicate a 2 min phase. The red and blue vectors in the circular plot (right) indicate the mean heading direction and vector length *r* of the corresponding phases shown in the flight tracks. The length of the vectors can vary between 0 (disoriented) and 1 (perfectly oriented). The inner dashed circle indicates a vector length of 0.2 and the perimeter of the plot a vector length of 1.

The use of a panoramic skyline

To investigate how butterflies orient with respect to a global, terrestrial cue, we presented the animals with a panoramic skyline with a variable height profile (Fig. 3). While the background above the horizon was set to blue light (emission peak at 458 nm and an intensity of ~4.61×10^{10} photons cm^{-2} s^{-1} per LED; panorama), the LEDs in the lower part were turned off. The butterflies' headings were recorded for 8 min while the position of the stimulus switched by 180 deg every 2 min. In a control experiment, the profile of the panorama was removed by switching off LEDs below an elevation of ~0 deg, which resulted in a flat horizon (flat panorama). To gain a deeper understanding of how the butterflies used the presented simulated panoramic skyline, compass orientation versus flight stabilization, we performed an experiment with a stationary grating of vertical stripes in blue (emission peak at approximately 458 nm; three columns of LEDs per stripe, spatial frequency of ~0.044 cycles deg^{-1}; experiment: grating) and black. Each blue LED had an intensity of ~4.61×10^{10} photons cm^{-2} s^{-1}. The flight performance of the butterflies was recorded for 4 min.

Combination of terrestrial and sun compass information

To answer the question of whether monarch butterflies combine different visual cues to increase their flight accuracy, we presented the panoramic skyline together with the bright green sun stimulus (experiment: panorama and sun; Fig. 4). Each butterfly's orientation performance was recorded for 8 min and the position of the panorama and sun was switched by 180 deg every 2 min. In a control experiment where the profile of the panorama lacked any bumps (i.e. was a flat horizon), the panoramic features were excluded but the sun was available (experiment: sun and flat panorama).

In an additional experiment, we investigated how the disappearance of a visual cue affects the butterflies' orientation performance (Fig. 5). We first allowed the butterflies to acclimate to the experimental conditions for 2 min (with the green sun and panorama available) as we noticed in the sun and panorama experiments (Figs 2E and 3C) that the orientation abilities of butterflies significantly changed over the first 2 min. In the subsequent 30 s, the butterflies were again presented with the combination of the panoramic skyline with variable heights and the green sun stimulus (combination). For the next 30 s, we excluded one of the stimuli [we either removed the sun stimulus or removed the peaks of the profile of the panorama (single cue)]. Half of the animals were first tested without a panorama (but with the sun), while half of the butterflies first experienced the panorama (without the sun). All butterflies experienced both stimuli again for an additional 30 s (combination) before the other stimulus that was present in phase 2 (either the simulated sun or the panorama) was removed (single cue) for a further 30 s. The order of the stimulus presentation (both cues/simulated sun, both cues/panorama) was pseudorandomized.

Data analysis

All data were analyzed in the software MATLAB (Version R2017b, MathWorks, Natick, MA, USA) using the CircStat toolbox (Berens, 2009). The experiments that took 8 min (green sun, blue sun, no cue, panorama, flat panorama, panorama and sun, sun and flat panorama; Figs 2–4) were divided into four phases of equal length, and all butterflies that stopped flying more than four times during the experiments were excluded from the analysis. The grating experiment (Fig. 3) was split into two phases (2 min each). Because this experiment lasted for only 4 min, butterflies that stopped flying more than two times were excluded from the analysis. This exclusion criterion was also used for the combination experiment (Fig. 5). Depending on the experiment, the data were divided into either 2 min (Figs 2–4) or 30 s (Fig. 5) phases.

To present the data with respect to the stimulus position, all heading directions were shifted in such a way that the simulated sun or a specific point of the panorama stimulus was positioned at 0 deg. For each butterfly, we calculated the flight trajectory (e.g. in Fig. 1B), and the mean vector within each 10 s bin and within a phase (2 min bins) (Figs 2–4). The mean direction μ of each butterfly within a phase was calculated (Fig. 1B). In the combination experiment, each phase lasted for 30 s instead of 2 min. To obtain the animal's performance on a finer scale in the combination experiment (Fig. 5), we calculated the vector length r within a window size of 1 s. This allowed us to register the effect of the disappearance of one cue on the animal's orientation ability within a very short time period. To avoid any misinterpretation of these r-values (they are higher than the r-values over 10 s or 2 min), we normalized all r-values to the highest r-value obtained in each flight. To further analyze the butterflies' performance in our flight simulators, we calculated the angular speeds of the butterflies. A highly oriented animal shows low angular speeds, usually caused by slow swinging around the heading direction. Highly disoriented animals exhibit high angular speeds, often caused by rapid rotation. The angular speed of individual butterflies was defined by calculating the absolute angular difference between two consecutive headings (Figs 3 and 5). To further test whether the butterflies followed a relocation of a stimulus, their change of heading was calculated by measuring the angular differences in the mean direction between two consecutive phases (Figs 2F and 3D,E). As each individual experienced three stimulus relocations over the 8-min flight (after 2, 4 and 6 min), we calculated the mean change of heading over three stimulus relocations in each animal.

Statistics

During our experiments, we noticed that many butterflies exhibited very poor performance in the first 2 min as compared with the remaining 6 min (e.g. green sun and blue sun; Fig. 2E). The time course of a butterfly's ability to keep a constant heading varied somewhat between experiments within the first phases compared with the following phases. To ensure that we compare the butterflies at a phase when they had adjusted to the experimental situation, we focused on the last phase of each experiment for the statistical evaluation. A possible bias of the heading directions towards a certain direction within this phase was tested with the non-parametric Moore's modified Rayleigh test (Moore, 1980). Furthermore, some butterflies performed poorly and failed to follow the change of the stimulus position. To compare the performance of the butterflies, we therefore calculated the mean r within the last 2 min phase of the control experiments plus the 95% confidence interval (no cue: r=0.1169; flat panorama: r=0.1194). We used a chi-square test to compare the proportion of butterflies above these thresholds. All animals that showed a lower directedness than these r-values were excluded from the comparison. The performance of the butterflies was statistically compared using a Kruskal–Wallis test for samples of different groups or using the Wilcoxon signed-rank test for comparison within the same group of butterflies (e.g. Fig. 5). The Mardia–Watson–Wheeler test was used to compare the heading directions of different butterfly groups.

RESULTS

The sun compass in butterflies

To study the orientation of monarch butterflies with respect to a simulated sun, we recorded the flight performance while the animals were tethered at the center of the LED flight simulator (Fig. 1A) and were presented with a green, bright light spot against a blue background as their only orientation reference. Many monarch butterflies, even though they were outside of their migratory phase, kept a constant heading direction with respect to this stimulus. When the stimulus's position was turned by 180 deg, these butterflies changed their heading accordingly (Fig. 2A, green

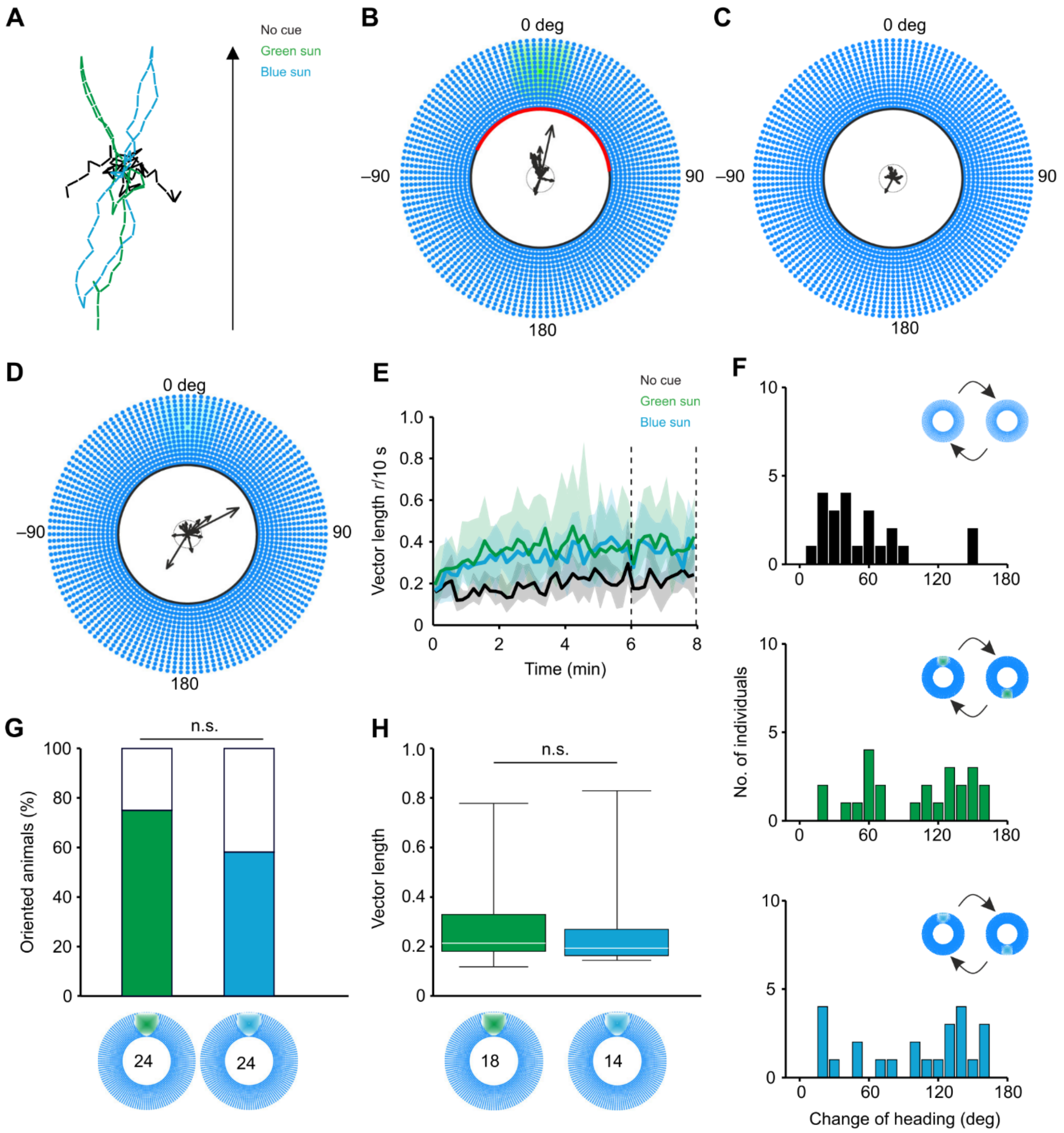

Fig. 2. The sun compass in monarch butterflies. (A) Flight trajectories of individual butterflies that viewed a bright green sun (green), a bright blue sun (blue) or no cue (black) as orientation reference. When the sun stimulus was relocated by 180 deg, the butterflies followed the change of the stimulus's position. Black arrow indicates the position of the sun stimulus or a specific point in the control scenery in the beginning of the experiment. (B–D) Orientation of butterflies with respect to a green sun (B; *N*=24), without any compass cues (C; *N*=22) or a blue sun (D; *N*=24). The mean vector for every butterfly was calculated over a 2-min phase. The inner circle of the plots indicates *r*=0.2. The red sector shows the circular standard deviation (s.d.) of the animals' significant group orientation. (E) The mean vector length *r* (bin size: 10 s) over entire experiments shows that the butterflies were better oriented with respect to a sun stimulus (green and blue curves) compared with the control condition without any directional information (black curve). Shaded areas indicate the 25–75% quantile. The vertical dashed lines indicate the 2-min section that was used to present the heading direction in B–D. (F) Histogram of heading changes after a 180 deg relocation of the stimulus (bin size: 5 deg) for the no cue (upper plot), the green sun (middle plot) and the blue sun (lower plot) experiments. (G) The number of 'oriented' butterflies was calculated by analyzing which animals showed a vector length *r*>0.1169 (mean + 95% confidence interval of the no cue experiment, i.e. the data shown in C) and was similar in both experiments (chi-square test: *P*=0.36, χ^2=0.84). (H) The mean vector length did not differ significantly between the experiments with the green and blue sun stimulus (Kruskal–Wallis test; *P*=0.43, χ^2=0.64). White horizontal lines indicate the median vector length. The boxes show the interquartile range and whiskers extend to the 2.5th and 97.5th percentile. n.s., *P*>0.05.

trajectory). On average, the butterflies chose headings towards the simulated sun (non-parametric Moore's modified Rayleigh test: P=0.002, R^*=1.46, μ=9 deg with respect to the simulated sun, $N_{\text{green sun}}$=24; Fig. 2B). Next, we switched the green stimulus LED to blue, so it was indistinguishable from all other LEDs in the arena. Unsurprisingly, the r-values, which describe the orientation precision of each butterfly across the 2-min phase, were significantly lower in the absence of the simulated sun (no cue; Fig. 2C) than when the sun stimulus was available (Kruskal–Wallis test: P=0.001, χ^2=10.59, $N_{\text{green sun}}$=24, $N_{\text{no cue}}$=22). This was also evident when we analyzed the flight directedness on a much finer temporal scale (Fig. 2E): the vector length r/10 s increased from on average 0.30±0.16 (mean±s.d.) over the first 2 min when the animals viewed the simulated sun and remained stable at a vector length of approximately 0.39±0.20 for the subsequent 6 min of flight (green sun; Fig. 2E). In contrast, the vector length remained relatively low (at 0.20±0.10) throughout the entire 8-min flight in the absence of any cue (no cue; Fig. 2E). Taken together, the improvement of orientation in the presence of a directional stimulus and the following of the stimulus show that non-migrating butterflies use the sun stimulus in our flight simulator for orientation.

To investigate whether the butterflies relied on the spectral or the brightness component of the sun stimulus for orientation, we presented the simulated sun as a bright, blue spot. Similar to what we observed with the green sun, butterflies were able to keep a directed course with respect to the blue sun and changed their heading when the stimulus was displaced by 180 deg (Fig. 2A, blue trajectory). The heading directions of the butterflies in the blue sun experiment were uniformly distributed across possible compass directions (non-parametric Moore's modified Rayleigh test: P=0.22, R^*=0.71, $N_{\text{blue sun}}$=24; Fig. 2D), showing no bias to any specific direction. The vector length r/10 s over the entire flight sequence exhibited a similar time course for the experiments with the green sun and blue sun (Fig. 2E). Although many butterflies kept a constant course in both experiments, we noticed that only a subpopulation of animals followed the 180 deg relocation of the stimulus [14 out of 24 (green sun) and 15 out of 24 (blue sun) showed a change in heading >90 deg; Fig. 2F]. The remaining butterflies did not change their heading as expected if they used the presented cues for orientation. To exclude any potential effects resulting from differences in the butterflies' behavioral state, we analyzed how many animals exhibited a higher r-value under conditions with a cue (green sun and blue sun) compared with the mean [plus 95% confidence interval (CI)] r-values when no visual cue (no cue) was available. Eighteen out of 24 butterflies (75%) presented with the green sun stimulus showed a higher mean vector length r than when the cue was absent (r>0.1169), while only 14 out of 24 animals (58%) showed a higher vector length in the blue sun experiment (Fig. 2G). These proportions of 'oriented' animals were not significantly different (chi-square test: P=0.36, χ^2=0.84, $N_{\text{green sun}}$=24, $N_{\text{blue sun}}$=24; Fig. 2G). The vector length r of these 'oriented' animals was similar in the green sun and blue sun experiments (Kruskal–Wallis test: P=0.43, χ^2=0.64, $N_{\text{green sun}}$=18, $N_{\text{blue sun}}$=14; Fig. 2H) and no significant differences in the heading directions between both groups were found (Mardia–Watson–Wheeler test: P=0.06, W=5.64, $N_{\text{green sun}}$=18, $N_{\text{blue sun}}$=14). This suggests that a sun stimulus that contains only brightness information elicits a similar ability to keep a constant heading as a stimulus that contains both spectral and brightness information.

The use of a panoramic skyline

Next, we presented a panoramic skyline to the animals with the panorama's profile consisting of smaller and higher bumps (Fig. 3A). The animals kept arbitrary headings with respect to the variable height panorama stimulus (non-parametric Moore's modified Rayleigh test: P=0.37, R^*=0.58, N_{panorama}=25; Fig. 3A). When the panorama was flat, i.e. the peaks of the panorama's profile were absent, we observed that none of the tested animals kept a constant heading (flat panorama, N=18; Fig. 3B). Although the length of mean vector r was similar when the panorama's profile exhibited variable heights (Fig. 3A) compared with when the panorama was flat (Kruskal–Wallis test: P=0.12, χ^2=2.41, N_{panorama}=25, $N_{\text{flat panorama}}$=18; Fig. 3B), the vector lengths r/10 s over the entire experiment were significantly longer with the panorama with variable heights as the compass cue (Kruskal–Wallis test: P<0.001, χ^2=13.53, N_{panorama}=25, $N_{\text{flat panorama}}$=18; Fig. 3C). This suggests that the panoramic skyline stimulus improves the butterfly's ability to maintain a directed course.

We also noticed that the butterflies did not change their headings when the visual scene was turned by 180 deg (Fig. 3D,E). In general, they did not keep a certain heading direction with respect to the panorama stimulus (as in the sun experiments) but rather constantly changed their headings over the course of the experiment (Fig. 3F). This opens up the possibility that the panorama might not be used for compass orientation but for flight stabilization. This is further supported by the observation that the rotational speed of the animals, i.e. their angular speed, was significantly lower when the panoramic features were available to the animals than when the panorama was flat (Kruskal–Wallis test: P<0.0001, χ^2=2633.71, N_{panorama}=25, $N_{\text{flat panorama}}$=18; Fig. 3G). To next study the butterflies' performance with respect to a visual stimulus that provides a strong rotational optic flow but lacks any directional information, we conducted an additional experiment in which we presented a stationary grating pattern to the butterflies (grating; N=21; Fig. 3H). Interestingly, the butterflies showed angular speeds up to approximately 540 deg s^{-1} in both panorama experiments (panorama and flat panorama). In contrast, the animals' angular speeds did not exceed 180 deg s^{-1} in the grating experiment (Fig. 3I). This demonstrates that optic-flow information is perceived by the monarch butterflies in this setup and plays an important role while the animals aim to keep a constant flight direction.

To compare the butterflies' orientation performance between the panorama and grating experiment, we first analyzed how many animals exhibited a higher r-value in these experiments than in the flat panorama (mean r+95% CI) experiment. Nine out of 25 animals (36%) showed a higher r-value in the experiment with the panorama, 12 out of 21 (57%) were higher in the grating experiment (Fig. 3J). The proportion of 'oriented' butterflies was the same in both experiments (chi-square test: P=0.26, χ^2=1.30, N_{panorama}=25, N_{grating}=21; Fig. 3J). The mean vector length r of the 'oriented' animals did not differ between both experiments (Kruskal–Wallis test: P=0.48, χ^2=0.51, N_{panorama}=9, N_{grating}=12; Fig. 3K). Nevertheless, we noticed that two butterflies exhibited a very high directedness with the variable height panorama (r>0.4; Fig. 3A,K), which was never observed in the grating pattern experiments (Fig. 3H,K).

Combination of terrestrial and sun compass information

To characterize orientation performance in the presence of both terrestrial and celestial cues, we presented the green sun combined with the panoramic skyline including a profile with variable heights (panorama and sun; N=24; Fig. 4A) or with a flat profile (sun and

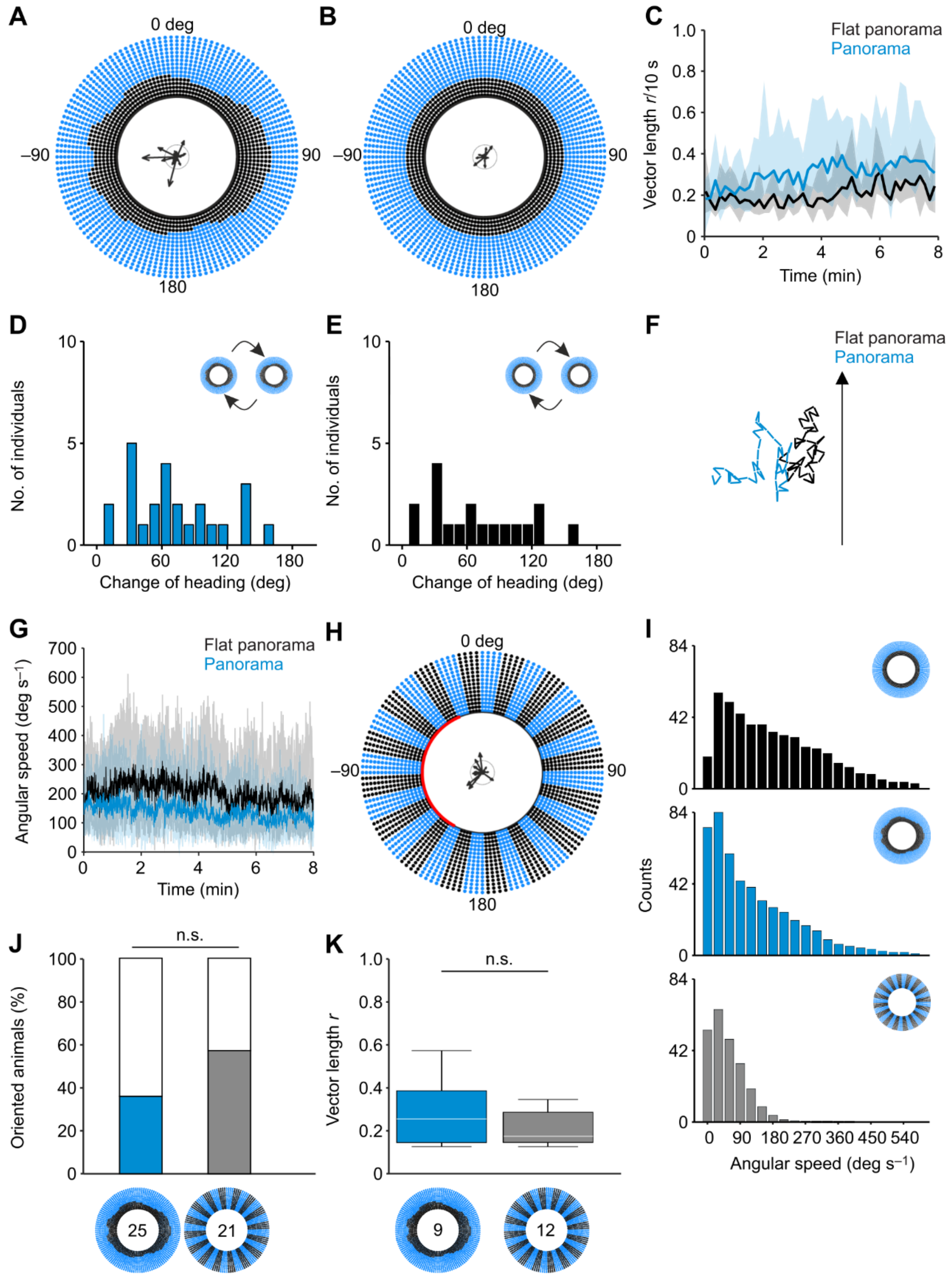

Fig. 3. See next page for legend.

flat panorama; *N*=25; Fig. 4B). When the panoramic skyline with variable heights was added to the scenery (panorama and sun), most of the animals kept a specific heading with a mean vector clockwise to the simulated sun (non-parametric Moore's modified Rayleigh test: $P<0.001$, $R^*=1.63$, $N=24$; $\mu=29$ deg with respect to the simulated sun; Fig. 4A). In contrast, the butterflies chose arbitrary headings in the sun and flat panorama experiment (non-parametric Moore's modified Rayleigh test: $P=0.34$, $R^*=0.60$, $N=25$; Fig. 4B). This effect was also observed in the experiment without the green sun (panorama; Figs 3A and 4C). The vector length *r*/10 s was

Fig. 3. Using a panoramic skyline for directed flight behavior. (A,B) Orientation of monarch butterflies with respect to a panoramic skyline with a variable height (A; $N=25$) or a flat profile (B; $N=18$). Dashed inner circle shows $r=0.2$. (C) Over the entire experiment, the mean vector length r (bin size: 10 s) was always higher when the panoramic skyline contained variable heights. Shaded areas indicate the 25–75% quantile. (D,E) Histogram of heading changes after a 180 deg relocation of the stimulus (bin size: 5 deg) for the panorama experiments with a variable height (D) and a flat profile (E). (F) Exemplary flight trajectories of one animal that flew with respect to a panoramic skyline with a variable height profile (blue trajectory) and one that oriented to a panoramic skyline with a flat profile (black trajectory). Black arrow indicates the position of a specific point in the visual scenery in the beginning of the experiment. (G) The angular speed of the animals over the 8 min flight. The angular speed decreased when the panoramic skyline with variable heights was visible (blue curve) compared with the flat panorama (black curve). The shaded area indicates the 25–75% quantile. (H) The orientation of butterflies with respect to a grating pattern ($N=21$). Dashed inner circle indicates a vector length of 0.2. The red sector shows the circular s.d. of the animal's group orientation. (I) The frequency of observed angular velocities (window size of each bin: 30 deg) when the butterflies had a flat panoramic skyline (upper plot, same data as G), the panorama with a variable height profile (middle plot, same data as G) or a grating pattern as orientation reference. (J) The number of 'oriented' butterflies was defined as $r>0.1194$ (which is the mean r+95% confidence interval of the flat panorama experiments, i.e. the data shown in B,C) and was similar between all experiments (chi-square test: $P=0.26$, $\chi^2=1.30$). (K) The vector length of the 'oriented' animals did not differ significantly between the experiments with the skyline panorama and the grating pattern as visual stimulus (Kruskal–Wallis test: $P=0.48$, $\chi^2=0.51$). White horizontal lines indicate the median vector length. The boxes show the interquartile range and whiskers extend to the 2.5th and 97.5th percentiles. n.s., $P>0.05$.

stable over the entire flight when both the sun and panorama were available (mean±s.d.=0.38±0.20; panorama and sun; Fig. 3D) and was significantly higher compared with the condition without the sun (mean±s.d.=0.30±0.19; panorama) (Kruskal–Wallis test: $P=0.005$, $\chi^2=8.07$, $N_{\mathrm{panorama}}=25$, $N_{\mathrm{panorama\ and\ sun}}=24$; Fig. 4D). However, the directedness did not differ between the panorama and sun and when the panorama's profile was flat (mean±s.d.= 0.35±0.20; sun and flat panorama) (Kruskal–Wallis test: $P=0.33$, $\chi^2=0.94$, $N_{\mathrm{panorama\ and\ sun}}=24$, $N_{\mathrm{sun\ and\ flat\ panorama}}=25$; Fig. 4D). To compare the performance of the butterflies, we calculated how many animals exhibited a higher directedness (r-value) in the sun and flat panorama and panorama and sun experiments compared with the mean r-value+95% CI ($r=0.1194$) in the flat panorama experiments (Fig. 3B). Irrespective of the panorama, more animals showed higher r-values as soon as the simulated sun was available (chi-square test: $P=0.002$, $\chi^2=9.48$, $N_{\mathrm{panorama}}=25$, $N_{\mathrm{panorama\ and\ sun}}=24$; $P=0.048$, $\chi^2=3.92$, $N_{\mathrm{panorama}}=25$, $N_{\mathrm{sun\ and\ flat\ panorama}}=25$; Fig. 4E). The performance of these 'oriented' animals did not show any significant differences (Kruskal–Wallis test: $P=0.61$; $\chi^2=0.99$, $N_{\mathrm{panorama}}=9$, $N_{\mathrm{panorama\ and\ sun}}=20$, $N_{\mathrm{sun\ and\ flat\ panorama}}=17$; Fig. 4F), which suggests that combining different visual cues does not necessarily help to improve the directedness of the butterfly's flight behavior.

The previous experiment did not allow us to test whether the butterflies registered both visual cues in their compass or whether they relied on the simulated sun as their only reference (while ignoring the panoramic skyline). We therefore performed an experiment in which we presented both cues (green sun and panorama; combination; Fig. 5A) to the butterflies in a first phase and subsequently withheld one of the cues (single cue; Fig. 5A) during a second phase (followed by an additional phase with both cues – combination – and a subsequent disappearance of the other cue – single cue – see Fig. 5A). Eighteen of 25 butterflies showed a performance with higher r-values in the two combination phases than in the flat panorama experiment (Fig. 3C). When analyzing the switch from the two visual stimuli to one cue in these 18 animals, we found that irrespective of which cue we turned off, this led to a significant decrease in the directedness r of the butterflies (Wilcoxon signed-rank test: $P<0.001$, $Z=4.33$, $N=18$ when the peaks of the panorama's profile disappeared; Fig. 5B, upper panel; $P<0.001$, $Z=3.71$, $N=18$ when the simulated sun disappeared; Fig. 5B, lower panel). Associated with this drop in the vector length, the angular speed increased when only one cue was available (Wilcoxon signed-rank test: $P<0.001$, $Z=-9.40$, $N=18$ when the peaks of the panorama's profile disappeared; $P<0.001$, $Z=-7.90$, $N=18$ when the simulated sun disappeared; Fig. 5C), further confirming that both cues are being registered by the butterflies. Interestingly, the disappearance of a specific cue had different effects in different animals. In several animals (eight out of 18), we found a drop in the vector length r when one of the cues – the simulated sun or the panorama – was excluded from the visual scenery (Fig. 5D, upper and middle panels). In other butterflies (10 out of 18), the disappearance of one of the cues did not have any effect on the directedness (Fig. 5D, lower panel), which indicates that they can dynamically switch from one to another as main orientation reference. Taken together, the data show that monarch butterflies can register multiple visual cues to keep a directed course. However, the relevance of these cues seems to differ in the tested animals.

DISCUSSION

The sun compass

Our experiments show that monarch butterflies – even when they are not in their migratory phase – use a green light cue (simulated sun) to keep a constant heading. This suggests that sun-compass orientation is not restricted to migration in this species. Whether non-migrating butterflies also shift their heading direction in a time-dependent manner to the simulated sun, as the migratory butterflies do to the real sun (Mouritsen and Frost, 2002; Froy et al., 2003), remains to be investigated.

Our results show that monarch butterflies sometimes prefer to choose a heading towards the simulated green sun (Fig. 2B), although in some experiments they took arbitrary headings (Fig. 4B). The latter is similar to the finding in the fruit fly *Drosophila melanogaster* that also maintains arbitrary headings (Giraldo et al., 2018) and suggests that they are able to perform compass orientation with respect to the sun stimulus. The heading choices towards the green sun in Fig. 2B may potentially result from a reduced ability of the butterflies to detect the sun stimulus in front of the bright blue background. In previous experiments, the sun was presented in front of a dark background (el Jundi et al., 2015b; Giraldo et al., 2018), whereas in our experiments the illuminated background led to a reduced contrast between the sun stimulus and the background (the sun stimulus was only two orders of magnitude brighter than the background). This may result in heading directions where the animals keep the stimulus frontally in their visual fields. This is in line with the arbitrary heading choices in Fig. 4B, where fewer blue LEDs were turned on and, thus, a stronger contrast between the sun and blue background was presented to the butterflies. It will now be interesting to test what headings the non-migrating butterflies prefer if we study them with respect to the real sun outdoors. But why do the butterflies, even when they are not in their migratory phase, keep constant headings in the flight simulator, and what is their behavioral state? Our current interpretation is that the butterflies exhibit an escape response and use the sun stimulus as a reference as has been shown in

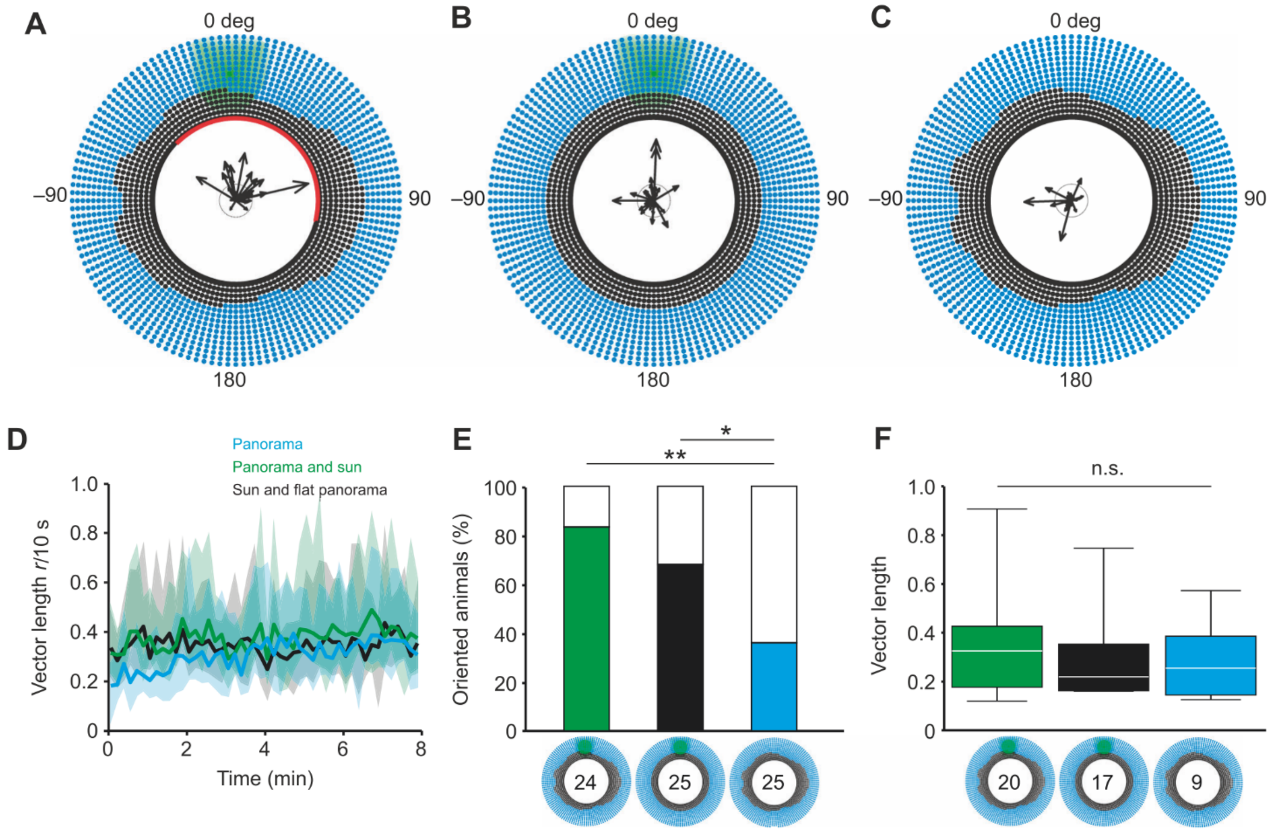

Fig. 4. Combining different visual cues for orientation. (A–C) The orientation of butterflies with respect to the panoramic skyline with a variable height (A; N=24) or a flat (B; N=25) profile combined with a green sun or the panoramic skyline alone (C; N=25, same data as shown in Fig. 3A). Dashed inner circle of the circular plots indicates an r-value of 0.2. The red sector in A indicates the s.d. of the mean group direction. (D) Over an 8-min flight, the mean vector length r was relatively high irrespective of whether only one of the cues, the variable height panorama (blue curve) or the sun (black curve), or both panorama and sun (green curve) were available. Shaded areas show the 25–75% quantile. (E) The number of animals with a vector length over r>0.1194 (which is the 95% confidence interval of the control experiment flat panorama). Independent of the panorama, more animals were 'oriented' when the green sun was added to the scenery (chi-square test: panorama and sun against panorama: P=0.002, χ^2=9.48; sun and flat panorama against panorama: P=0.048, χ^2=3.92). (F) The orientation performance of the 'oriented' animals did not differ significantly between the experiments with one of the cues (blue box plot=panorama only; black box plot=sun only) or both cues available (green box plot) (Kruskal–Wallis test: P=0.61, χ^2=0.99). White horizontal lines represent the median vector length r. The boxes show the interquartile range and whiskers extend to the 2.5th and 97.5th percentile. n.s., P>0.05.

non-migrating butterflies outdoors previously (Kanz, 1977). An alternative explanation is that the butterflies' goal, on their search for food, is to disperse into a new niche, a behavior that is well established for butterflies under natural conditions (Felt, 1925; Stevens et al., 2010).

Our experiments show that monarch butterflies use a sun stimulus that contains only brightness information in a very similar way as a stimulus that contains both spectral and brightness information. This suggests that intensity information of the sun can be used by the butterflies to keep a directed course, which is in line with an electrophysiological study that indicates a wavelength-independent neural coding of the sun in the monarch butterfly's brain (Heinze and Reppert, 2011). In nature, owing to a different ratio of shorter (UV light) and longer (green light) wavelengths of light between the solar and anti-solar hemisphere, the direction of the sun can be determined based on a spectral contrast (Coemans et al., 1994; el Jundi et al., 2014). Whether monarch butterflies can use this spectral information, similar to what has been shown for bees (Brines and Gould, 1979; Edrich et al., 1979; Rossel and Wehner, 1984) and dung beetles (el Jundi et al., 2015a, 2016), remains to be shown in further experiments. In bees, a green light cue is interpreted as the sun while a UV light cue is treated as a patch of the sky somewhere in the anti-sun direction (Brines and Gould, 1979; Edrich et al., 1979; Rossel and Wehner, 1984). Unfortunately, our current LED stimulus does not allow testing for this in monarch butterflies as the stimulus lacks any UV light. This could also explain why the butterflies in our control experiments (Figs 2C and 3B) could not use their inclination compass, as it relies on light in the UV to violet range (Guerra et al., 2014). Thus, to fully determine whether monarch butterflies use spectral information for orientation, we aim to additionally present UV light in our flight simulator in the future.

The use of a panoramic skyline

Monarch butterflies rely on a sun compass (Mouritsen and Frost, 2002; Stalleicken et al., 2005), and potentially also use terrestrial cues to keep a desired heading direction in a similar way as it has been shown in the past for other insects (Cartwright and Collett, 1983; Collett and Land, 1975; Fleischmann et al., 2018; Lehrer and Collett, 1994). Ants and wasps are well-known to use the panoramic skyline as an orientation reference during homing (Graham and Cheng, 2009a,b; Philippides et al., 2011; Reid et al., 2011; Narendra et al., 2013; Narendra and Ramirez-Esquivel, 2017; Stürzl et al., 2016). Calvert (2001) observed a change in the monarch butterflies' migratory direction as soon as they reached the mountains of the

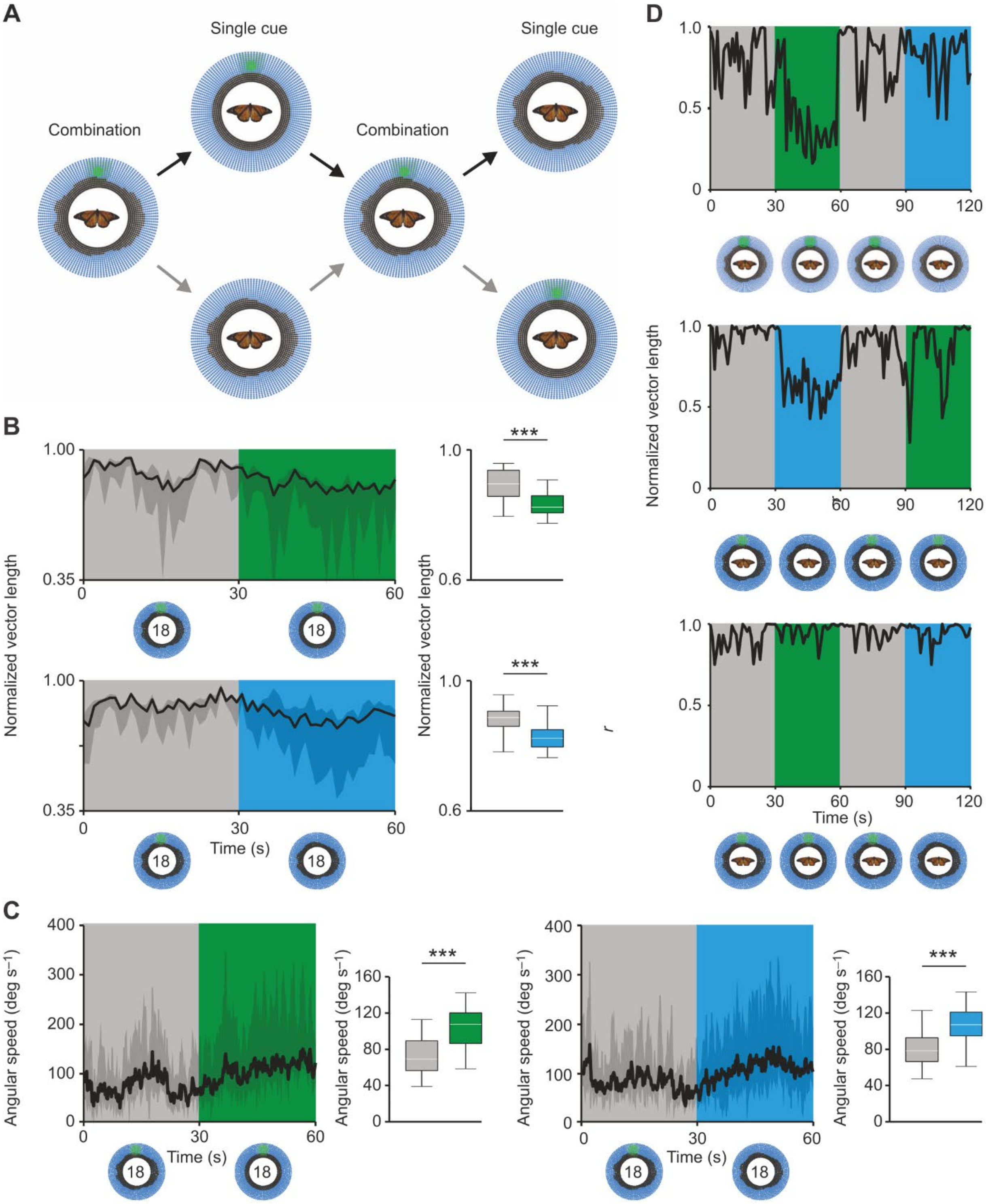

Fig. 5. See next page for legend.

Sierra Madre Oriental. The author suggested that the butterflies might use the beneficial wind conditions generated by the mountain ranges to migrate toward Mexico (Calvert, 2001). Similarly, it was suggested in another study that the Rocky Mountains act as a physical barrier and funnel the butterflies towards Mexico (Mouritsen et al., 2013). In these cases, the animal's compass can obtain a higher robustness for the maintenance of the migratory direction by combining and matching the sun's position with the terrestrial scenery. It will therefore be very interesting to test whether terrestrial cues play a major role in the context of migration.

Terrestrial cues might especially be relevant if the animals orient in their natural habitat in their non-migratory phase, e.g. during

Fig. 5. Monarch butterflies can use a combination of different cues for orientation. (A) Schematic illustration of the experimental procedure. We tested the use of different cues by presenting both cues (panorama and simulated sun, phase 1) to the butterflies and subsequently removing one of the cues (phase 2), either the panorama with a variable height profile (black arrow) or the sun (gray arrow). In the third phase, we presented both cues to the butterflies before one cue was withheld in the fourth phase. Each phase lasted for 30 s. (B) On average, the butterflies showed a significant decrease in their directedness when either of the cues was excluded. Left plot: normalized vector length (per 1 s) when the panorama (upper panel) or the simulated sun (lower panel) was excluded after 30 s of flight. Gray shaded areas in the left plots represent the 25–75% quartile. Right plot: box plots of the averaged normalized vector length (same data as in left plots). The normalized vector length dropped significantly when the bumps of the panoramic skyline (Wilcoxon signed-rank test: upper plot, $P<0.001$, $Z=4.33$) or the green sun (lower plot, $P<0.001$, $Z=3.71$) disappeared. ***$P<0.001$. (C) The angular speed increased when one cue (left panel, variable height panorama; right panel, green sun) disappeared (same data as in C). The angular speed increased significantly when the green sun (Wilcoxon signed-rank test: left box plot, $P<0.001$, $Z=-9.40$) or the panorama (right box plot, $P<0.001$, $Z=-7.90$) was suddenly the only available orientation reference. Gray shaded areas in the left plots represent the 25–75% quartile. Box plots: white horizontal lines represent the median vector length. The boxes indicate the interquartile range and whiskers extend to the 2.5th and 97.5th percentiles. ***$P<0.001$. (D) The normalized vector length (per 1 s) of oriented, individual butterflies is shown over the 2 min experiments. Individual butterflies used different cues for orientation. Some butterflies relied on one of the cues, panorama (upper panel) or the simulated sun (middle panel), and their performances decreased when this cue was excluded. A few butterflies used both cues and showed a high orientation throughout the entire experiment (lower panel).

foraging. We therefore presented a dark silhouette of a panoramic skyline to the butterflies as findings in ants suggest that the contrast of objects against the sky is important for the animals' orientation (Graham and Cheng, 2009a). In our experiments, most of the butterflies used the presented panorama to keep a certain heading only over a short time period and did not follow a 180 deg relocation of the stimulus. Apart from directional information, a panoramic skyline provides an animal with rotational optic flow information, which can be used by insects for positional control (Wolf and Heisenberg, 1990). Although it is very difficult to unravel how exactly the butterflies interpreted the panorama stimulus, our data suggest that they mainly used it for flight control. Nevertheless, some individuals showed well-oriented flights when presented with the panorama with high r-values (Fig. 3A). These were not observed when the animals had optic flow (but no distinct cue) for orientation (Fig. 3H), which indicates that these animals used the panorama for compass orientation. Whether the use of the panorama as a compass cue is dependent on the distinctness of the features of the panorama's profile is currently not known, but will be the focus of our research in the future. Furthermore, it will be interesting to determine whether they can store and memorize a desired heading with respect to the panoramic scene, a similar matching strategy to the one that has been shown in ants (Lent et al., 2010).

Combination of multiple cues

We presented the green sun stimulus in combination with the panoramic skyline to study how the butterflies use a visual scene that mimics a combination of celestial and terrestrial information. We found that the presentation of both cues did not lead to a more directed flight performance (Fig. 4), as has been shown for the combination of multimodal cues in ants, moths and dung beetles (Dacke et al., 2019; Dreyer et al., 2018a; Huber and Knaden, 2017; Müller and Wehner, 2007). The ability of the butterflies to keep a directed heading over larger time periods was dominated by the presence of the simulated sun, which is in line with observations in migrating butterflies. This suggests that the sun is the main orientation cue for monarch butterflies (Stalleicken et al., 2005). Nevertheless, we found that in the absence of the sun or the panorama the directedness of the butterflies was affected (Fig. 5). Some animals used both cues during flight, while other individuals relied predominantly on the simulated sun or the panoramic skyline as a reference. This indicates that the butterfly's compass is capable of combining and weighting different visual cues, similar to what has been shown in ants and dung beetles (el Jundi et al., 2015b, 2016; Huber and Knaden, 2017). This is also similar to findings in the migrating Bogong moth, which uses different modalities – the Earth's magnetic field and dominant visual cues – for orientation (Dreyer et al., 2018a). When these cues were set in conflict, several moths were disoriented while other individual moths remained well oriented. This is in line with our results that reveal a strong interindividual variability in the weighting of different orientation cues in lepidopterans and raises the question of what mechanism lepidopterans in general, and butterflies specifically, use to combine different cues in their compass. One mechanism that butterflies could use is to store multiple cues of a scene in a snapshot (with respect to the desired heading direction) and to match it to the current view, a strategy that is used by orienting dung beetles (el Jundi et al., 2016; Dacke and el Jundi, 2018). Similar to these beetles (el Jundi et al., 2015b), we know that the central complex acts as an internal compass for the butterfly's migration (Heinze and Reppert, 2011). Thus, this brain region likely plays a major role in the integration of sun and terrestrial compass information as it provides the neuronal substrate that allows a flexible combination of different cues in the insect's compass (Fisher et al., 2019; Kim et al., 2019; Seelig and Jayaraman, 2015). The results here show that non-migrating monarch butterflies can keep constant headings with respect to a visual scene based on skylight and terrestrial cues, similar to what migrating butterflies do during their annual journey. This suggests that the central complex controls orientation at any stage of the butterfly's life, allowing us to study the neural mechanisms of the butterfly's compass in detail, not only during their migration but also while they are in their non-migratory phase.

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Competing interests
The authors declare no competing or financial interests.

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Data availability
Raw data can be downloaded from https://doi.org/10.6084/m9.figshare.12129984.v1. Analysis scripts can be obtained from the corresponding author upon request.

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5 Manuscript III:

Stimulus-dependent orientation strategies in monarch butterflies

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Stimulus-dependent orientation strategies in monarch butterflies

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Abstract

Insects are well-known for their ability to keep track of their heading direction based on a combination of skylight cues and visual landmarks. This allows them to navigate back to their nest, disperse throughout unfamiliar environments, as well as migrate over large distances between their breeding and non-breeding habitats. The monarch butterfly (*Danaus plexippus*) for instance is known for its annual southward migration from North America to certain trees in Central Mexico. To maintain a constant flight route, these butterflies use a time-compensated sun compass for orientation which is processed in a region in the brain, termed the central complex. However, to successfully complete their journey, the butterflies' brain must generate a multitude of orientation strategies, allowing them to dynamically switch from sun-compass orientation to a tactic behavior toward a certain target. To study if monarch butterflies exhibit different orientation modes and if they can switch between them, we observed the orientation behavior of tethered flying butterflies in a flight simulator while presenting different visual cues to them. We found that the butterflies' behavior depended on the presented visual stimulus. Thus, while a dark stripe was used for flight stabilization, a bright stripe was fixated by the butterflies in their frontal visual field. If we replaced a bright stripe by a simulated sun stimulus, the butterflies switched their behavior and exhibited compass orientation. Taken together, our data show that monarch butterflies rely on and switch between different orientation modes, allowing the animal to adjust orientation to its actual behavioral demands.

Introduction

Orientation in space is an essential ability for animals to find food, escape from predators, or return to their nest. To achieve this, insects exhibit a number of different orientation mechanisms, ranging from the simple straight-line orientation of dung beetles (Dacke et al., 2021; el Jundi et al., 2019) to more complex behaviors such as path integration of ants and bees (Collett and Collett, 2000; Heinze et al., 2018) or long-distance migration of lepidopterans (Grob et al., 2021; Hu et al., 2021; Merlin and Liedvogel, 2019; Warrant et al., 2016). One striking example of a migrating insect is the monarch butterfly (*Danaus plexippus*) (Reppert and de Roode, 2018; Reppert et al., 2016). Each fall millions of these butterflies migrate from the northern USA and Canada over more than 4,000 km to their overwintering habitat in Central Mexico. To keep a constant direction over this enormous distance, these animals rely on the sun for orientation (Froy et al., 2003; Heinze and Reppert, 2011; Mouritsen and Frost, 2002; Reppert, 2006). In combination with time-of-day information from circadian clocks in the brain (Sauman et al., 2005) and/or the antennae (Guerra et al., 2012; Merlin et al., 2009) this allows the butterflies to maintain a directed course throughout the day. Beside the sun, additional cues, such as the celestial polarization pattern (Reppert et al., 2004) or the Earth's magnetic field (Guerra et al., 2014; Wan et al., 2021) seem to play a role during the migration but their relevance for the butterfly's compass is still not fully understood (Stalleicken et al., 2005).

As in other insects, the central complex of monarch butterflies serves as an internal compass during spatial orientation (el Jundi et al., 2014; Heinze and Reppert, 2011; Heinze et al., 2013; Pfeiffer and Homberg, 2014). Compass neurons in this brain region are sensitive to multiple simulated skylight cues (Heinze and Reppert, 2011; Nguyen et al., 2021) and encode the animal's heading with respect to a sun stimulus (Beetz et al., 2021). As shown previously, a sun stimulus – represented by a green light spot – can be employed in behavioral laboratory experiments in monarch butterflies (Franzke et al., 2020). Similar experiments in the fruit fly *Drosophila melanogaster* demonstrated that these insects exhibit a menotactic behavior with respect to a simulated sun. This means that the fruit fly maintains any arbitrary heading relative to the sun (Giraldo et al., 2018). Interestingly, closed-loop experiments showed that as soon as the activity of central-complex neurons was genetically deactivated, the flies kept the simulated sun in their frontal visual field, resembling vertical stripe fixation behavior (Giraldo et al., 2018). This attraction behavior does not depend on whether the flies are confronted with a bright stripe on a dark background or the inverted visual scene (Maimon et al., 2008). Although the biological function of the fly's attraction behavior is not fully understood, it is speculated that the flies interpret this cue as a landing or feeding site (Maimon et al., 2008). Whether monarch butterflies adjust their orientation strategy depending on the visual stimulus is not known. However, to successfully display a large repertoire of behaviors, the orientation network in the butterfly's brain needs to possess the capacity to flexibly switch between different orientation circuitries that may operate in parallel in the brain. This would, for instance, allow a flying butterfly to change from compass orientation based on skylight cues to attraction based on a visual landmark or an odor plume similar to what has been found for homing desert ants (Buehlmann et al., 2013).

To study the monarch butterflies' behavioral repertoire, we recorded the orientation behavior of flying butterflies, tethered at the center of an LED-flight simulator, while we provided different visual cues (*dark stripe*, *bright stripe*, and *sun stimulus*) to the animals. We found that the butterflies used the dark stripe for flight stabilization based on optic-flow information. A bright stripe on the other hand evoked a simple attraction behavior towards the stimulus. In contrast, a simulated sun was used by the butterflies to maintain a constant angle with respect to the stimulus. We furthermore found that the butterflies switched between compass orientation and attraction behavior during flight. Taken together, our results show that monarch butterflies display different orientation modes that allow them to dynamically switch between different behaviors while navigating through their environment.

Material and Methods

Experimental animals

Pupae of the monarch butterfly (*Danaus plexippus*) were ordered from Costa Rica Entomology Supply (butterflyfarm.co.cr) and kept in an incubator (HPP 110 and HPP 749, Memmert GmbH+Co. KG, Schwabach, Germany) at 25°C and 80% relative humidity and under a 12:12 h light:dark cycle. After the animals eclosed, the adult butterflies were transferred to a flight cage inside a separate incubator (I-30VL, Percival Scientific, Perry, IA, USA) with a 12 h:12 h light:dark cycle. While the relative humidity was constant at about 50%, the temperature was set to 25°C during light phases and 23°C during dark phases. Feeders inside the flight cage were filled with 15% sucrose solution and provided ad libitum food to the butterflies.

Preparation

We used female and male adult butterflies (2-3 weeks after eclosion) and prepared them in the morning prior to each experiment. We removed the scales of the butterflies' thorax and glued (multi-purpose impact instant contact adhesive, EVO-STIK, Bostik Ltd, Stafford, UK) a tungsten stalk (0.508×152.4 mm, Science Products GmbH, Hofheim, Germany) to the dorsal side. After preparation, the animals were individually kept in clear plastic containers with access to 15% sucrose solution and transferred to a dark chamber for at least three hours. For each experiment, a new group of butterflies was used except for the experiments in Fig. 1 (*dark stripe*, *no cue*, *bright stripe*) where 20 animals experienced at least two stimulus conditions.

Flight simulator

We used a flight simulator similar to the ones described previously (Dreyer et al., 2018a; Dreyer et al., 2018b; Dreyer et al., 2021). To record the heading directions of individual butterflies, the tungsten wire on the animals' thorax was connected to an optical encoder (E4T miniature Optical Kit Encoder, US Digital, Vancouver, WA, USA). This allowed the butterflies to rotate at the center of the flight simulator and freely choose any heading. Butterflies that stopped flying for more than four times during an experiment were excluded from the study. The heading direction of the animals was recorded with an angular resolution of 3 deg and a temporal resolution of 200 ms using a data acquisition device (USB4 Encoder Data Acquisition USB Device, US Digital, Vancouver, WA, USA) and a computer with the corresponding software (USB1, USB4: US Digital, Vancouver, WA, USA). To present visual stimuli to the butterflies, the inner surface of the flight simulator was equipped with a circular array of 2048 RGB LEDs (128*16 APA102C LED Matrix, iPixel LED Light Co.,Ltd, Baoan Shenzhen, China) or green high power LEDs (LZ1-00G102, OSRAM, San Jose, CA, USA). A custom written python script was used to control the color and intensity of all LEDs of the LED arena via a raspberry pi (Raspberry Pi 3 Model B, Raspberry Pi Foundation, UK).

Stimuli

In all experiments we presented the butterflies with one or multiple different visual stimuli. To produce them, the intensity and color of each LED of the arena was adjusted as summarized in the following table:

Stimulus symbol	*dark stripe* 	*bright stripe* 	*sun stimulus* 	*green stripe* 
Stimulus proportion	Three LED columns (43.3 deg height and 8.4 deg width)	Three LED columns (43.3 deg height and 8.4 deg width)	One LED at an elevation of ~23 deg	Three LED columns (43.3 deg height and 8.4 deg width)
Stimulus color	none	Blue with an emission peak at 458 nm	Green with an emission peak at 520 nm	Green with an emission peak at 520 nm
Stimulus intensity	none	$\sim 1.65 \times 10^{13}$ photons cm^{-2} s^{-1} for the whole stripe	$\sim 1.0 \times 10^{13}$ photons cm^{-2} s^{-1}	$\sim 1.65 \times 10^{13}$ photons cm^{-2} s^{-1} for the whole stripe
Background color	Blue with an emission peak at 458 nm	none	none	none
Background intensity	$\sim 4.61 \times 10^{10}$ photons cm^{-2} s^{-1} for each LED	none	none	none

Table 1. Properties of the different presented stimuli. The table summarizes the proportion, color, and intensity of each stimulus as well as the color and intensity of the background of the LED arena.

Orientation with respect to one cue

In the first set of experiments, we presented one cue (stripe or simulated sun) as an orientation reference to the butterflies. In all experiments, the heading direction of a single animal was recorded over eight minutes. To ensure that the butterflies used the displayed cue for orientation, we turned the visual scenery by 180 deg every two minutes and studied if the animals followed the relocation of the cue. We alternated the start position of the stimulus between 0 deg and 180 deg, starting at 0 deg for half of the animals and at 180 deg for the other half.

First, we investigated whether monarch butterflies can use a landmark for orientation by setting all LEDs of the arena to blue while three LED columns were turned off which generated a dark stripe on a bright background (Fig. 1A; experiment: *dark stripe*). 28 butterflies were then individually connected via the tungsten wire to the encoder at the arena's center and were allowed to orient by changing their heading direction with respect to the landmark (Fig. 1B-D first panel; Fig. 2B, 2D, 2F). As a control, we also performed an experiment with 22 individuals, in which the animals did not perceive any visual cue for orientation. Therefore, all LEDs were set to blue (experiment: *no cue*; Fig. 1B-D second panel; Fig. 2A; same data as in Franzke et al., 2020). Next, we inverted the visual scenery by turning all LEDs off with the exception of three LED columns which were set to blue. We recorded the headings of 22 butterflies presented with this bright stripe (experiment: *bright stripe*; Fig. 1B-D third panel; Fig. 2C, 2E, 2G).

Finally, we investigated which orientation strategy monarch butterflies display when they were flying with respect to a simulated sun. Previous studies revealed that a green light cue is interpreted as the direction towards the sun by several insects (Edrich et al., 1979; el Jundi et al., 2015; Rossel and Wehner, 1984). Therefore, we presented a simulated sun to 20 animals by turning one bright green LED on (experiment: *sun stimulus*; Fig. 3A&B, 3E&F). To test if the spectral content of our stimuli (green sun stimulus vs. blue stripe) had an impact on the orientation behavior of the butterflies, we repeated the experiments with the bright stripe with 20 butterflies. This time, the stripe changed its color every two minutes of flight from green to blue and *vice versa* (Fig. 3G-H). Again, the position and color of the stimulus was alternated between each butterfly. This means a quarter of the animals first experienced a green stripe at 0 deg (green stripe 0/blue stripe 180/green stripe 0/ blue stripe 180 deg) while a quarter of the butterflies started with a green stripe at 180 deg first (green stripe 180/ blue stripe 0/green stripe 180/blue stripe 0 deg). The remaining animals perceived the blue stripe at either 0 deg (blue stripe 0/green stripe 180/blue stripe 0/green stripe 180 deg) or 180 deg first (blue stripe 180/green stripe 0/blue stripe 180/green stripe 0 deg).

In a final step, we investigated if the butterflies changed their orientation behavior when we changed the stimulus during a butterfly's directed flight. Therefore, we repeated the experiment with the blue vs. green stripe with 33 animals. However, instead of changing the color, this time we changed the appearance of the stimulus presenting a bright, green stripe for one minute followed by a sun stimulus for another one-minute phase (or *vice versa*; Fig. 5A). The animals' orientation was recorded over two minutes and the position and stimulus order (sun stimulus to stripe, stripe to sun stimulus) was alternated for each butterfly.

Orientation within an ambiguous scenery

In the second set of experiments, we presented two identical stimuli to the butterflies, that were set 180 deg apart from each other. In all experiments, the position of the stimuli was relocated, this time by 90 deg, every two minutes for a total of eight minutes. We alternated the start position of the stimulus between 0&180 deg and 90&270 deg, starting at 0&180 deg for half of the animals and at 90&270 deg for the other half. In the first experiment, we tested the orientation behavior of 18 butterflies with respect to two dark vertical stripes. All LEDs of the arena were turned blue and to generate the stripes, two sets of three LED columns were turned off (experiment: *two dark stripes*; Fig. 4A, 4D first panel). Next, we recorded the headings of 18 butterflies presented with two bright stripes on a dark background. For this experiment, all LEDs were turned off and the stripes were generated by turning two sets of three LED columns blue (experiment: *two bright stripes*; Fig. 4B, 4D second panel). In addition, we tested 19 butterflies with respect to two artificial sun stimuli. Therefore, two LEDs at an elevation of about 23 deg were turned green (experiment: *two sun stimuli*; Fig. 4C; 4D third panel).

Data analysis

Heading directions were calculated by importing the data into the software MATLAB (Version R2017b, MathWorks, Natick, MA, USA) and analyzing it using the CircStat toolbox (Berens, 2009). As we changed the position of the visual stimulus every two minutes, we divided the 8-minute flights of the butterflies into four equal 2-minute sections and the 4-minute flights (stripe vs. sun stimulus) into two sections. The flight trace of each butterfly (e.g., Fig. 1B), the mean vector within each ten-second bin (Figs. 1C&F, 3B&D) and within a section (two-minute bins, Figs. 3G&I, or one-minute bins, 5A) was calculated. As the animals' directedness can increase over the first four minutes of an experiment (Franzke et al. 2020), we focused on the butterfly's flight performance in the last two flight sections (i.e., the last four minutes) of each experiment. Thus, the change of direction was measured as the angular difference between the mean heading directions taken during the last two flight sections (Fig. 1D, 3E&H). We then related all recorded heading angles relative to the stimulus position (stimulus position = 0 deg) and calculated the mean heading vector over the last four minutes (Fig. 2C&F, 3F, right panel). To analyze if the animals maintain a directed flight course over a shorter time period, we counted the number of ten-second bins that exceeded a directedness of $r = 0.249$ (which is the mean vector strength + 95% confidence interval for 10s bins in the last four minutes of the no cue experiment). For another detailed analysis of the heading distribution, we counted how often each animal kept every angle (in three deg bins) relative to the stimulus position. We defined the heading direction with the most counts as the animals' preferred angle and normalized the number of counts of all other headings to this value. The normalized heading counts were plotted in relation to the stimulus (stimulus position = 0 deg) to generate a heatmap (e.g., 2A). For a better visualization of a bimodal or unimodal distribution of headings, we plotted the normalized heading counts in relation to the animals preferred heading (Fig. 4D). To test the butterfly's performance in the presence of the ambiguous stimuli, we analyzed the flight trajectories over the last four minutes with a temporal resolution of two seconds. We then selected all flight sections in which the animals maintained a straight flight (<45° change in heading) over at least four seconds. We then categorized the subsequent change in flight direction according to the angular change in heading: if a butterfly changed its heading direction between 140 and 220 deg before returning to a straight flight course, this was categorized as a 'half turn'. In contrast, a 'full turn' was defined when an animal changed its heading by more than 90 deg and the next phase of oriented flight deviated by less than 40° from the original direction. Based on these data, we calculated a turning index by subtracting the number of ‘full turns’ by the number of ‘half turns’ and by dividing this value by the sum of all turns. Thus, animals with a positive turning index performed more ‘full turns’ while a negative turning index represents more ‘half turns’. All butterflies that performed neither ‘full turns’ nor ‘half turns’ were excluded from this analysis.

Statistics

The non-parametric Moore’s Modified Rayleigh test (Moore, 1980) was used to test for a bias of heading directions within a flight sector. Furthermore, we compared the heading directions of different butterfly groups with the Mardia-Watson-Wheeler test. In the experiment where we changed the color of the bright stripe, we used the v-test to test whether the butterflies kept the same heading after the stimulus manipulation. To compare the performance of the butterflies, we first calculated the mean vector strength within the last four minutes and statistically compared them using a Kruskal-Wallis test for samples of different groups, the Wilcoxon signed-rank test for samples of the same individuals, or a linear mixed model ANOVA when the same butterflies participated in more than one condition. To test whether the mean vector strength in ten-second bins increased over time, we used the Wilcoxon signed-rank test. Additionally, we compared the number of ten-second bins with a vector strength above 0.249 of different animals using a Kruskal-Wallis test or a linear mixed model ANOVA. To test whether butterflies presented with two bright stripes, or two sun stimuli differed in their amount of ‘full turns’ vs. ‘half turns’, we compared the turning indices of both experimental groups with the Kruskal-Wallis test.

Results

Landmark orientation to a vertical stripe

To investigate how monarch butterflies use a local landmark for orientation, we performed flight-simulator experiments in which individual animals were tethered at the center of an LED arena (Fig. 1A). We first presented a dark vertical stripe on a blue background to the butterflies. Although the animals only weakly oriented, we observed sequences in which they kept a certain heading over a short time (Fig. 1B, first panel;

video S1). This was different from the butterflies' behavior in a scene without any cue (Fig. 1B, second panel). To quantify if the butterflies more often maintained constant heading directions when they had the vertical stripe as a reference, we calculated the vector strength of the mean orientation vector for each 10 s segment of the entire flight for each animal (Fig. 1C). This value ranges from 0 to 1 and indicates how well a butterfly maintained its flight course (with 0 being completely disoriented and 1 being perfectly directed). When the butterflies had the vertical stripe for orientation, they showed a vector strength of about 0.2 ± 0.1 in the first 10 s of their flight that increased significantly to a vector strength of 0.5 ± 0.2 in the last 10 s of the flight (P=0.007, Z=-2.710, Wilcoxon signed-rank test; N=28; Fig. 1C, *dark stripe*). Without any orientation cue, the butterflies showed a vector strength of 0.2 ± 0.1 (bin size: 10 s) that did not increase throughout the experiment (Fig 1C, N=22, *no cue*). This performance was significantly worse than when the vertical stripe was available as an orientation cue comparing the vector strength over the last two phases (P<0.001, F=22.788, linear mixed model ANOVA; bin size: 4 min; Fig. 1E). The higher vector strength with the vertical stripe was a result of significantly more oriented phases with r>0.249 (see methods; P<0.001, F=13.450, linear mixed model ANOVA; bin size: 10 s; $N_{\textit{no cue}}$=22, $N_{\textit{dark stripe}}$=28; Fig. 1F).

We also analyzed if the butterflies changed their heading when the position of the dark vertical stripe was moved by 180 deg and found that 18 out of 28 butterflies followed the stimulus relocation (directional change >90 deg; Fig. 1D, first panel). In contrast, most of the butterflies (18 of 22) tested without an orientation reference did not change their heading in a meaningful way (Fig. 1D, second panel). In summary, our data suggest that the butterflies can use a dark stripe to maintain a directed flight course.

We next tested how the butterflies use a bright stripe for orientation by inverting the visual scenery (i.e., a bright vertical stripe on a dark background). In contrast to the flight behavior with the dark stripe, many butterflies kept a constant heading over a longer time window or even over the entire 8-minute flight (Fig. 1B, third panel; video S2). This higher orientation performance was also reflected in the animals' vector strength which significantly increased from about 0.3 ± 0.1 at the beginning to a maximum of 0.65 ± 0.3 at the end of the experiment (P<0.001, Z=-3.230, Wilcoxon signed-rank test; bin size: 10 s; N=22; Fig. 1C, *bright stripe*). The vector strength of the last 4 minutes of flight was significantly higher than when the butterflies had the dark vertical stripe for orientation (P<0.001, F=13.440, linear mixed model ANOVA; bin size: 4 min; Fig. 1E). Similarly, the number of oriented phases was significantly higher with the bright stripe as an orientation reference (P=0.012, F=6.844, linear mixed model ANOVA; bin size: 10s; Fig. 1F). As expected, most of the animals (20 of 22) followed the relocation of the bright stripe (Fig. 1D, third panel) when we changed the position by 180 deg.

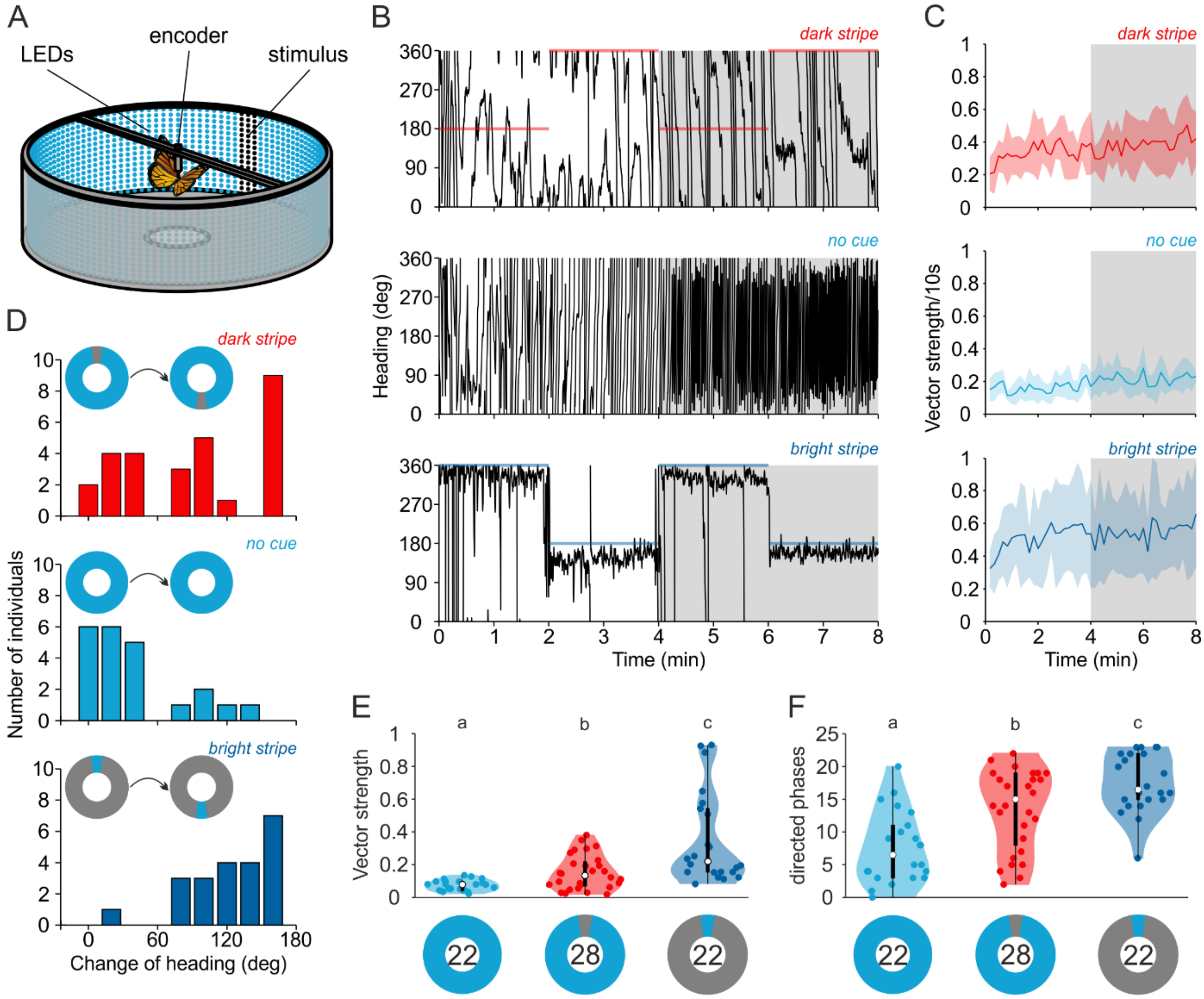

Fig. 1. Landmark orientation to a vertical stripe. (A) Schematic illustration of a monarch butterfly tethered at the center of a flight simulator equipped with 2048 RGB-LEDs. While presenting visual stimuli to the butterflies, their heading directions were recorded using an

optical encoder. (B) Flight trace of exemplary butterflies that were flying in the LED arena with respect to a dark stripe on a blue background (*dark stripe*), with all LEDs turned blue (*no cue*), or with a bright, blue stripe on a dark background as orientation reference (*bright stripe*). Colored, horizontal lines indicate the position of the vertical stripe at either 0 or 180 deg. The gray boxes indicate the 4-min sections that were used for further analysis. (C) The mean vector strength r (bin size: 10 s) over the entire eight-minute experiments shows that the butterflies' orientation performance increased over time. The animals were better oriented when a dark stripe was added to a bright background (*dark stripe*, first panel; *no cue*, second panel) and performed the best when a bright stripe was presented (*bright stripe*, third panel) as measured by an increase in vector strength. Shaded areas indicate the 25–75% quantile. The gray boxes indicate the 4-min sections that were used for further analysis. (D) Change of heading (bin size: 20 deg) between the last two phases of an experiment (indicated by the gray boxes in B) when the stimulus was relocated by 180 deg. Fewer animals changed their heading when no cue was available (*no cue*), but most animals followed a relocation of the stripes by more than 60 deg (*dark stripe*, *bright stripe*). (E) The vector strength (bin size: 4 min) was significantly higher when a dark stripe was added to a blue background ($P<0.001$, $F=22.788$, linear mixed model ANOVA) but not as high as when a bright stripe was displayed (*dark stripe* against *bright stripe:* $P<0.001$, $F=13.440$, linear mixed model ANOVA). White dots indicate the median vector strength. The black boxes show the interquartile range and thin black lines extend to the 1.5 x interquartile range. Colored dots show the individual data points and shaded area represents their density. Letters indicate a significant difference between the tested groups. (F) Significantly more directed phases (vector strength >0.249; bin size: 10s) were observed when a dark stripe was added to the blue background ($P<0.001$, $F=13.450$, linear mixed model ANOVA). This number increased when a bright stripe ($P=0.012$, $F=6.844$, linear mixed model ANOVA) was presented. Plot conventions as in E.

To gain insights into why the butterflies' performance was different between the two experiments (*bright vs. dark stripe*), we next analyzed the heading directions of butterflies within the two sceneries (Fig. 2). Interestingly, animals that were tested either without a cue (Fig. 2A) or with a dark stripe (Fig. 2B) headed in all possible directions. Calculating the mean direction for each butterfly within the last four minutes relative to the dark stripe showed that the butterflies maintained arbitrary heading directions ($P=0.996$, $R^*=0.038$, non-parametric Moore's modified Rayleigh test; $N_{dark\ stripe}=28$; Fig. 2D). However, as no butterfly maintained its direction over a longer flight sequence with the dark stripe, we studied the heading directions of the animals on a finer scale and selected only flight sequences in which the butterflies maintained a stable heading over a time window of 10 seconds. Even when we studied this, we found that the butterflies' short-term headings were randomly distributed ($P=0.506$, $Z=0.681$, Rayleigh test; bin size: 10 s; Fig. 2F), suggesting that they did not keep headings towards the stimulus. This was different from the butterflies' behavior when a bright stripe was presented (Fig. 2C). Here, we found that most of the well-oriented animals flew in the direction of the bright stripe ($P=0.006$, $R^*=1.296$, non-parametric Moore's modified Rayleigh test; $N_{\textit{bright stripe}}=22$; Fig. 2C&E), suggesting that the animals were attracted by the stimulus. This stripe attraction was also observable when we analyzed stable heading directions over short flight sections ($P<0.001$, $Z=43.542$, Rayleigh test; bin size: 10 s; Fig. 2G). Taken together, these results suggest that monarch butterflies display different behavioral strategies depending on the contrast between a vertical stripe and its background. While a dark stripe leads to several short phases of constant headings in arbitrary directions, a bright stripe allows the butterflies to maintain constant headings towards the stripe over long phases.

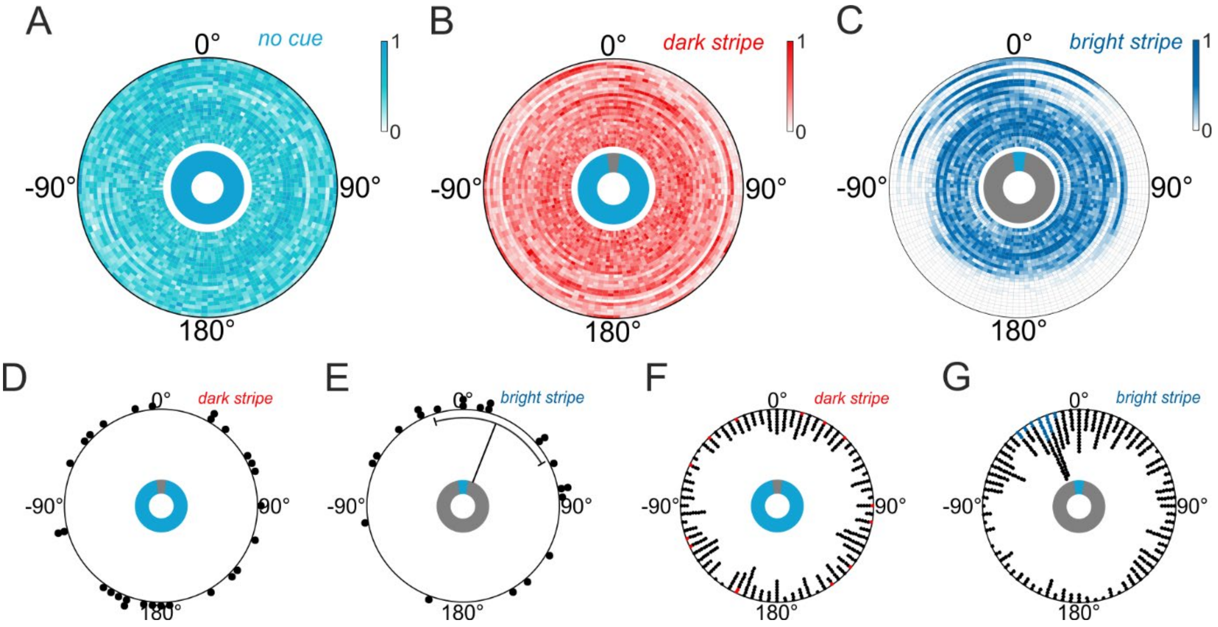

Fig. 2. Different orientation strategies depending on the contrast of the vertical stripe. (A-C) Orientation of individual butterflies in a flight simulator either (A) without visual reference

(*no cue*; N=22), (B) with a dark stripe on a bright background (*dark stripe*; N=28), or (C) to a bright stripe on a dark background (*bright stripe*; N=22) as orientation cue. The heat maps show the counted and normalized headings relative to a stimulus at 0 deg (bin size: 3 deg). Each ring represents the heading of one butterfly. The rings are sorted by increasing vector strength starting at the center. (D&E) Mean heading direction relative to a dark stripe (D) of the same butterflies as in (B). The mean flight direction to a bright stripe (E) of the same butterflies as in (C). Each dot in the circular plots represent the mean vector of one individual. (F&G) The directed phases (vector strength >0.249; bin size: 10 s) in the experiment with the (F) dark stripe are not clustered in any specific direction (P=0.506, Z=0.681, Rayleigh test). Red dots indicate the directed phases of an exemplary butterfly in arbitrary directions (P=0.774, Z=0.257, Rayleigh test). In the experiment with a bright stripe (G), most directed phases were found in the direction of the stimulus (P<0.001, Z=43.542, Rayleigh test). Blue dots indicate the directed phases of an exemplary butterfly in the direction of the bright stripe (P<0.001, Z=22.623, Rayleigh test).

Compass orientation with respect to a sun stimulus

We next wondered how monarch butterflies use a simulated sun for orientation. We therefore conducted an experiment with a green light spot as a simulated sun stimulus. Animals tested with respect to this stimulus kept constant headings over the entire experiment (Fig. 3A; video S3), although the butterflies directedness (as measured by the vector strength) significantly increased over time, from about 0.3 ± 0.1 at the beginning of the flight up to a maximum of 0.7 ± 0.3 at the end of the experiment (P=0.005, Z=-2.800, Wilcoxon signed-rank test; N=20; bin size: 10 s; Fig. 3B). The vector strengths over the last four minutes of the butterflies that oriented with the sun stimulus were in the same range as the ones that had the bright vertical stripe for orientation (Kruskal-Wallis test: P=0.801, χ^2=0.06; bin size: 4 min; Fig. 3C). Similarly, the number of oriented phases were not significantly different between the sun-stimulus and the bright-stripe experiment (P=0.129, χ^2=2.31, Kruskal-Wallis test; bin size: 10 s; Fig. 3D). Although most of the individuals (13 of 20) changed their heading by more than 90 deg when we changed the position of the sun stimulus by 180 deg (Fig. 1E), they did not keep this stimulus in their frontal visual field. Thus, the butterflies' heading directions were uniformly distributed (P= 0.130, R*= 0.825, non-parametric Moore's modified Rayleigh test; N=20; Fig. 3F). This suggests that monarch butterflies can maintain any desired compass direction with respect to a sun stimulus. This difference between how butterflies treated the sun stimulus and the bright stripe was not a consequence of a difference in the spectral content (blue stripe vs. green sun): when we changed the stripe color (from green to blue and *vice versa*) every two minutes, the butterflies showed well-oriented flights, irrespective of the stripe color (Fig. 3G&I) and did not change their heading relative to the bright stripe (P=0.001, u=3.047, v-test, expectation: 0 deg; Fig 3H). In contrast, the mean direction of butterflies tested with a green stripe differed significantly from the sun-stimulus heading distribution (P=0.02, W=7.823, Mardia-Watson-Wheeler test). This indicates that the butterflies ignore the spectral content of the cue and are attracted by the brightness of the stripe while a sun stimulus is used for compass orientation.

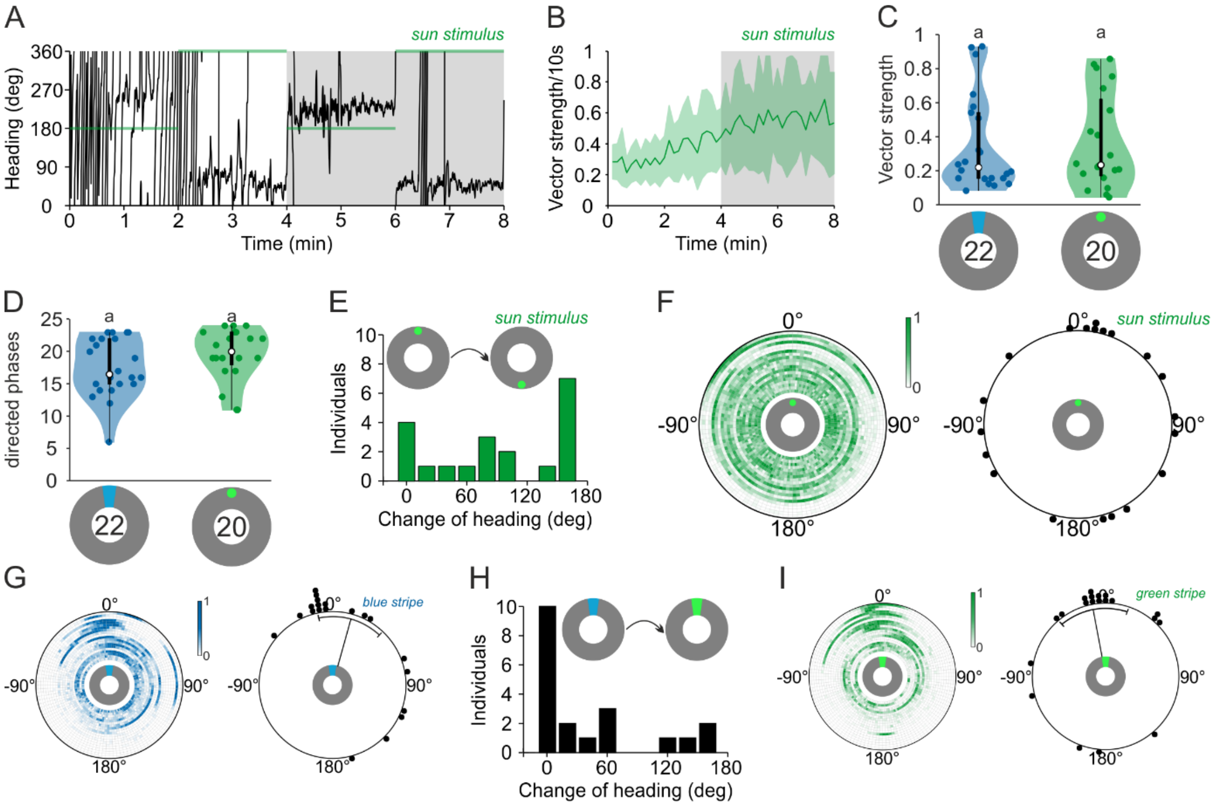

Fig. 3. Compass orientation with respect to a sun stimulus. (A) Flight trace of one exemplary butterfly that was flying in the LED arena with respect to a simulated sun. Colored, horizontal lines indicate the position of the sun stimulus at either 0 or 180 deg. The gray boxes indicate the 4-min sections that were used for further analysis. (B) The mean vector strength *r* (bin size: 10 s) over the entire eight-minute experiments shows that the butterflies were well oriented

and increased their orientation performance over time. Shaded areas indicate the 25–75% quantile. The gray boxes indicate the 4-min sections that were used for further analysis. (C) The orientation performance (bin size: 4 min) did not differ when presenting either a bright stripe or a sun stimulus (P=0.801, χ^2=0.06, Kruskal-Wallis test). White dots indicate the median vector strength. The black boxes show the interquartile range and thin black lines extend to the 1.5 x interquartile range. Colored dots show the individual data points and shaded area represents their density. Letters indicate a significant difference between the tested groups. (D) The number of oriented phases in animals flying with respect to a bright stripe was similar to the butterflies exposed to a simulated sun (P=0.129, χ^2=2.31, Kruskal-Wallis test). Plot conventions as in C. (E) Change of heading (bin size: 20 deg) between the last two phases of an experiment (indicated by the gray boxes in A) when the stimulus was relocated by 180 deg. Most of the animals changed their heading with the displacement of the visual stimulus. (F) Orientation of butterflies (N=20) flying with respect to a simulated sun. The heat map (left panel) shows the counted and normalized headings relative to the sun at 0 deg (bin size: 3 deg). Each ring represents the heading of one butterfly. The rings are sorted by increasing vector strength starting at the center. The mean headings of the same butterflies were directed in arbitrary directions (right panel). Each dot in the circular plots represent the mean vector of one individual. (G-I) Orientation of butterflies (N=20) when the color of a bright stripe was changed from blue (G, *blue stripe*, left panels) to green (I, *green stripe,* right panels) and *vice versa*. Independent of the spectral component the butterflies flew in the direction of the bright stripe. Plot conventions as in F. The black lines in the circular plots indicate the mean and the circular standard deviation in the direction of the stripes in both experimental groups. The butterflies did not change their heading (H, bin size: 20 deg) when we changed the color of the stripe (P<0.001, u=3.047, v-test).

Orientation in an ambiguous scenery

Our previous experiments suggest that monarch butterflies may exhibit different orientation strategies depending on the appearance of a visual stimulus: they likely use a dark vertical stripe to maintain constant courses over short flight periods, while a bright stripe evokes an attraction behavior. In contrast, a simulated sun is used for a menotactic behavior, i.e., for compass orientation. Interestingly, compass orientation requires the activity of the central-complex network in fruit flies, which is not necessary for an attraction towards a stripe (Giraldo et al., 2018). To investigate in more detail whether the butterflies use different visual orientation strategies and if the central complex is likely involved in coding them, we next performed experiments within ambiguous visual scenes (*two dark stripes*, *two bright stripes*, *two sun stimuli*; Fig. 4). We expected that the butterflies will maintain a distinct compass heading within such ambiguous sceneries if the heading-direction network of the central complex controls the orientation behavior (Beetz et al., 2021). When we provided two dark stripes as landmarks to the butterflies, their performance resembled the performance with one dark stripe. They showed short sections of straight flights in all possible heading directions that were interrupted by rapid rotations (Fig. 4A; N=18). Again, these findings support our observation that monarch butterflies use the dark stripe/s for flight stabilization rather than for compass orientation. When the butterflies oriented with two bright stripes, they maintained a constant heading towards one of the stripes. However, they frequently switched their fixation between the stripes by changing their heading by ~180 deg (example highlighted in dark gray in Fig. 4B, left panel). Consequently, the flight bearings were clustered around 0 and around 180 deg (Fig. 4B, right panel) which resulted in a bimodal distribution of heading directions relative to the positions of the stripes (Fig. 4D, second panel; N=18). When the butterflies were provided with the two simulated suns, they maintained arbitrary headings similar to the situation with one sun stimulus (Fig. 4C, left panel). This confirms our observation that they employ compass orientation with respect to light spots (Fig. 4C, right panel). However, we also noticed that the butterflies returned to their original bearing or headed into the opposite direction when they deviated from their course. This led to a bimodal distribution of heading directions with the second peak being less pronounced than in the two-bright-stripe experiment (Fig. 4D, third panel; N=19). To quantify if the butterflies more often returned to their original bearing when they viewed the two suns, we calculated a turning index for every butterfly. A negative turning index indicated a higher amount of 180 deg (half) turns while a positive turning index marked a higher ratio of returns to the original bearing (full turns). We found that the turning index was significantly higher with the two suns than with the two bright stripes ($P = 0.004$, $\chi^2 = 8.48$, Kruskal-Wallis test; $N_{\textit{two bright stripes}} = 17$, $N_{\textit{two sun stimuli}} = 15$; Fig. 4E). This suggests that the butterflies return to their original bearing more often when they had the two suns for orientation, a behavior that is expected if the heading-direction network of the butterfly's central complex controls the flight direction.

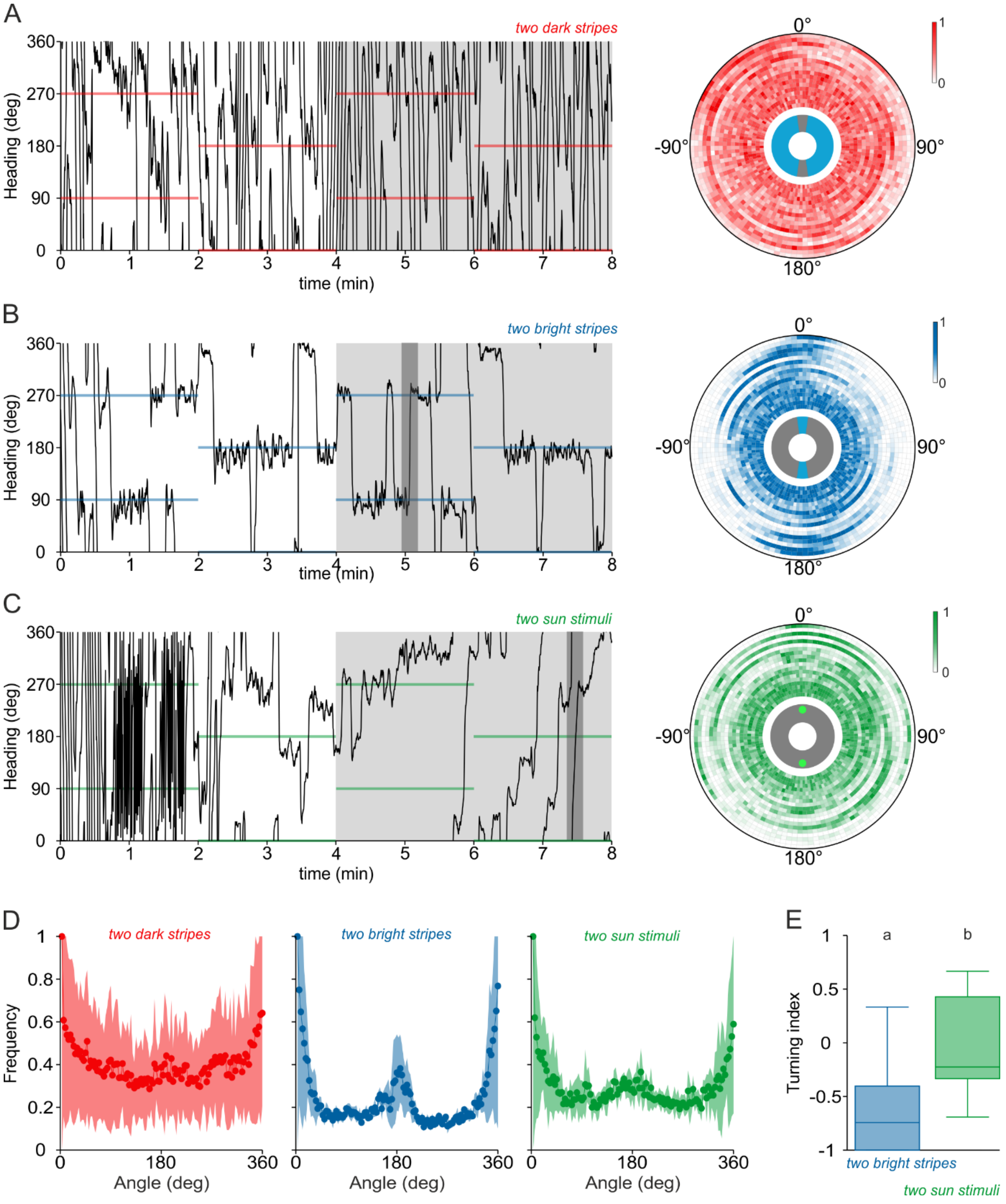

Fig. 4. Orientation in an ambiguous scenery. (A-C) Orientation of butterflies with respect to two dark stripes on a bright background (A, *two dark stripes*; N=18), two bright stripes on a

dark background (B, *two bright stripes*; N=18), or two sun stimuli (C, *two sun stimuli*; N=19). Left panels show exemplary flight trajectories of the experiments. Colored lines indicate the position of the visual stimuli at 90 and 270 deg or 0 and 180 deg. The light gray boxes indicate the 4-min sections that were used for further analysis. The dark gray part in B indicates a 'half turn' and the dark gray part in C indicates a 'full turn'. Right panels: The heat maps show the counted and normalized headings relative to the stimuli shifted at 0 deg and 180 deg (bin size: 3 deg). Each ring represents the headings of one butterfly. The rings are sorted by increasing vector strength starting at the center. (D) The mean frequency of angles relative to the preferred heading of each butterfly flying with two dark stripes (first panel), two bright stripes (second panel) or two sun stimuli (third panel) as orientation reference. Butterflies perceiving two bright stripes showed a sharp second peak at 180 deg. A much wider and less high secondary peak was found at 180 deg when the animals were presented with two sun stimuli. Dots and lines represent the mean frequency and shaded areas indicate the 25–75% quantile. (E) The turning indices were calculated by dividing the number of 'full turns' minus the number of 'half turns' by the sum of all turns and differed significantly between animals tested with two bright stripes or two sun stimuli ($P=0.004$, $\chi^2=8.48$, Kruskal-Wallis test). Horizontal lines indicate the median vector length. The boxes show the interquartile range and whiskers extend to the 2.5th and 97.5th percentiles. Letters indicate a significant difference between the tested groups.

Compass orientation vs stripe attraction

As our previous experiment suggests that the butterflies' orientation modes depended on the stimulus properties, we next wondered if the butterflies rapidly switch their orientation behavior if we changed the visual scene from a sun to a stripe stimulus (and *vice versa*). Again, when we presented a bright stripe to the butterflies, they fixated the stimulus in their frontal visual field (Fig. 5A, left panel). Interestingly, when we changed the stimulus to a simulated sun instead, the butterflies changed their heading direction and adopted arbitrary bearings with respect to the sun stimulus (Fig. 5A, right panel). The headings taken with respect to the sun stimulus were significantly different from the headings with respect to the bright stripe ($P=0.002$, $W=12.63$, Mardia-Watson-Wheeler test). Taken together, this shows that the butterflies can flexibly change their orientation strategy from compass orientation to stripe attraction during flight.

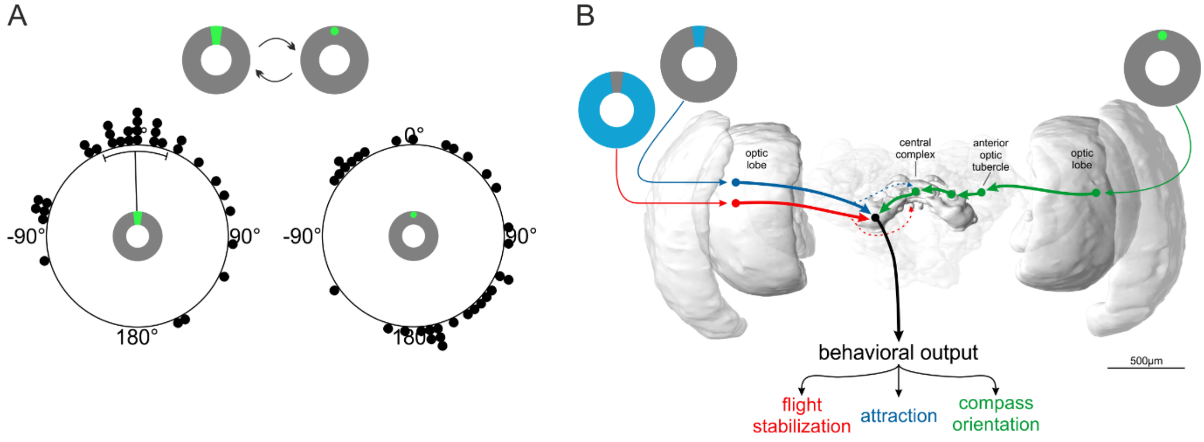

Fig. 5. Neuronal network controlling the switch between different orientation strategies. (A) Orientation of monarch butterflies (N=33) in a flight simulator when a bright stripe is replaced by a sun stimulus and *vice versa*. The animals switched from an attraction behavior in the direction of a bright stripe (left panel) to arbitrary directions with respect to the sun (right panel). Each dot in the circular plots represent the mean vector of one individual. The black lines indicate the mean and the circular standard deviation of the animals' significant group orientation. (B) Schematic illustration of the proposed different neuronal pathways resulting in different orientation strategies. While visual information about landmark cues (red and blue pathway) is directly transferred from the optic lobes to the lateral accessory lobes, information of the position of the sun (green pathway) is first integrated in the animals' internal compass system, the central complex, by passing the anterior optic tubercle and the bulb. Compass information from the central complex is sent to the lateral accessory lobe. From there, behavioral output is driven via descending neurons (black arrow) resulting in different orientation strategies depending on the visual input. The brain of the monarch butterfly is adapted from Heinze and Reppert (2012) and created via https://insectbraindb.org (Heinze et al., 2021).

Discussion

We here tested the ability of monarch butterflies to use different visual stimuli to maintain a directed flight course and found that they exhibit different orientation modes that depend on the stimulus identity. While the butterflies used the dark stripe to stabilize their flight, they exhibited a strong attraction to the bright stripe. In contrast to these rather simple strategies, a simulated sun evoked compass orientation. This suggests that different strategies operate in parallel in the brain (Fig. 5B) which allows monarch butterflies to effectively adapt their orientation strategy to a certain behavior by dynamically switching to the most appropriate strategy during flight.

Orientation to local cues

A bright stripe triggered an attraction behavior in monarch butterflies. We interpret this behavior as a brightness-based flight approach with the intention to leave the current setting and access a new environment similar to what has been found in navigating orchid bees (Baird and Dacke, 2016). This would also be in line with our observation that the behavior does not seem to be affected by the stripe's spectral information. However, instead of centering the bright stripe accurately in their frontal visual field, many butterflies kept the stripe slightly to their left/right vertical body axis. This indicates that the butterflies rely on the edge between the stripe and the background to sustain a constant heading, similar to what has been shown in walking Lucilia flies (Osorio et al., 1990). The stripe fixation of the butterflies that we described here is well in line with the results reported for tethered-flying *Drosophila* that are also attracted by a bright stripe (Giraldo et al., 2018; Maimon et al., 2008). However, the fly's positive taxis seem to be dependent on the behavioral or locomotory state. Thus, walking fruit flies can also adopt arbitrary headings with respect to a bright stripe (Green et al., 2019). Interestingly, in flying and walking fruit flies (Götz, 1987; Horn and Wehner, 1975; Strauss and Pichler, 1998) and other insects, such as flying locusts (Baker, 1979; Robert, 1988) or naïve walking ants (Buehlmann et al., 2020), a dark stripe also elicits stripe fixation. In contrast, monarch butterflies used the dark stripe to occasionally maintain a bearing over short phases in a random direction. This result is similar to what has been reported for monarch butterflies in a more complex visual scene, where they had the panoramic skyline for orientation (Franzke et al., 2020). Interestingly, in the same study the butterflies showed a similar behavior when they experienced a grating pattern – providing rotational optic flow – as the only visual input in a flight simulator (Franzke et al., 2020). Such rotational optic flow can provide an animal with directional information relative to a visual cue to perform compensatory steering and to keep a certain bearing (Wolf and Heisenberg, 1990; Zeil, 1996; Zeil et al., 2008). Thus, instead of using the vertical dark stripe to maintain a desired heading over an entire flight, our data suggest that the butterflies use optic-flow information to stabilize their heading over short flight sequences. In summary, a dark stripe evokes a different behavior in monarch butterflies than a bright stripe, which stands in contrast to *Drosophila*. It will be interesting to observe in the future at which visual angle of the bright stripe the butterflies will switch to an attraction behavior, and at which stripe width flight control will dominate the orientation behavior in monarch butterflies.

Sun compass orientation

When we presented a simulated sun to the butterflies, they kept arbitrary headings relative to the stimulus. This menotactic behavior is in line with what has been reported for other insects such as fruit flies (Giraldo et al., 2018) or dung beetles (Byrne et al., 2003). In theory, menotaxis can be carried out by a simple, vision-based retina matching of the current and remembered sun position similar to how many insects can use the profile of a panoramic skyline for orientation (Cartwright and Collett, 1983; Collett, 1992; Junger, 1991; Lent et al., 2010; Wehner and Räber, 1979). However, when we provided the butterflies with two simulated suns set 180 deg apart as orientation references, they returned to their original bearing during flight, which shows that they compute a distinct heading direction with respect to the ambiguous visual scene. This observation suggests that they do not only rely on the azimuth of the sun stimulus for orientation, but their orientation mechanism requires the involvement of the activity of a multisensory heading-direction network. This raises the question of what exactly defines the green light spot as a compass cue. In a recent paper, the butterflies' flight headings were directed towards the sun stimulus when the elevation of the sun stimulus was set to a low elevation of about 5 deg (Franzke et al., 2020). Even though the contrast between the background and the sun stimulus in Franzke et al. (2020) might have led to these heading choices, it opens up the possibility that the elevation of the sun stimulus is a critical parameter to induce compass orientation. In addition, for maintaining a certain heading direction, compass orientation also requires the network to memorize the desired direction (Grob et al., 2021; Honkanen et al., 2019). Whether the monarch butterfly can develop a long-term memory for a direction relative to the sun stimulus, as shown in the fruit fly (Giraldo et al., 2018) awaits to be investigated. Similarly, our future studies will focus on the use of the sun stimulus in the context of migration. Rather than adopting arbitrary headings, we expect that migratory monarch butterflies keep directed courses with respect to the sun stimulus that would guide them to the migration destination. Moreover, as the butterflies employ a time-compensated sun compass during their migration (Merlin et al., 2009; Mouritsen and Frost, 2002), we will next study if the heading to the sun stimulus will be adjusted according to the time-of-day. Taken together our findings show, that monarch butterflies use a sun stimulus for compass orientation, a strategy that allows them to maintain any arbitrary heading with respect to the sun during dispersal or in a distinct southward direction when they are in their migratory stage.

Neuronal network behind orientation

Our experiments suggest that the butterfly brain generates different orientation strategies but how is this accomplished at the neuronal level? As the butterflies used the dark stripe for flight control, the neuronal basis for it likely lies in the motion vision center, the lobula plate of the optic lobe (Meier and Borst, 2019; Ullrich et al., 2015). Although some optic-flow information is integrated into the central complex in locusts and bees (Rosner et al., 2019; Stone et al., 2017), the relevant information for flight control is directly transferred to the thoracic ganglia via descending pathways (Suver et al., 2016). In fruit flies, attraction does not require the activity of the central complex (Giraldo et al., 2018). This is well in line with our results from the 2-bright stripe experiment which points towards a coding of directional information without the association of a multisensory heading-direction network in monarch butterflies. Thus, the basis for the attraction to a bright stripe might also be based on the motion-vision network that is directly connected to descending neurons, as suggested in a recent model (Fenk et al., 2014) (Fig. 5B). In contrast, the butterflies resolved the ambiguity of the visual scene, when we instead presented two suns as stimuli for orientation. This matches recordings from the heading-direction network in the butterfly central complex that encodes an explicit heading based on multisensory inputs if confronted with a similar 2-sun stimulus (Beetz et al., 2021). Thus, our behavioral data suggest that the central complex encodes the sun stimulus, which is also in line with the sensitivity of central complex neurons to a green light spot (Heinze and Reppert, 2011; Nguyen et al., 2021). Recent results suggest that the central complex compares the actual heading direction with the desired direction (Green et al., 2019; Stone et al., 2017). By encoding the desired migratory direction, the butterfly's central complex is likely taking a central role in the migration and is the region in the brain where time-of-day information becomes relevant for sustaining the migratory southward direction. We therefore propose that compass orientation is processed by the central complex, while stripe attraction and flight control seem to rely on reflexive pathways without the involvement of a higher brain center (Fig. 5B). Our results here show that the butterflies can switch between compass orientation and attraction. Information from the central complex is sent to the lateral accessory lobe and further to the posterior protocerebrum in monarch butterflies (Heinze et al., 2013) where it might converge with the attraction and flight control pathways (Fig. 5B). Interestingly, recent results in the fruit fly suggest that descending neurons can generate different steering commands based on different input pathways (Rayshubskiy et al., 2020). This suggests that the reliance on different orientation strategies might also be weighted and governed by descending neurons in monarch butterflies, which allows butterflies to rapidly switch their orientation strategy during flight. This enables them to flexibly switch from a long-distance system during dispersal or migration to a short-distance orientation strategy such as the attraction to their host plant.

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Data availability

Raw data can be downloaded from https://figshare.com/articles/dataset/_/17082701. Analysis scripts can be obtained from the corresponding author upon request.

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Supplementary information

Video S1. Demonstration of a butterfly tethered at the center of a flight simulator and flying with respect to a dark stripe on a bright background. The position of the stimulus was relocated by 180 deg every two minutes.

Video S2. Demonstration of a butterfly tethered at the center of a flight simulator and flying with respect to a bright stripe on a dark background. The position of the stimulus was relocated by 180 deg every two minutes.

Video S3. Demonstration of a butterfly tethered at the center of a flight simulator and flying with respect to a bright green light spot as sun stimulus. The position of the stimulus was relocated by 180 deg every two minutes.

6 General discussion

The monarch butterfly is a fascinating insect known for its long-distance migration from northern USA and southern Canada to Central Mexico. On their more than 4.000 km long journey, they were found to use external reference cues such as the position of the sun (Froy et al., 2003; Merlin et al., 2009; Mouritsen and Frost, 2002) to maintain their course. Further studies suggest that the butterflies use in addition polarized light (Reppert et al., 2004), celestial gradients (Stalleicken et al., 2005), the Earth's magnetic field (Guerra et al., 2014), and specific mountain ranges (Calvert, 2001) to successfully maneuver towards Central Mexico. In contrast to this, the orientation ability of the summer generations and non-migratory populations of monarch butterflies is only poorly understood. Although they perform only short flights (Calvert, 2001; Zhu et al., 2009), they need to disperse from their place of origin to increase their individual's success in finding food, mating partners or a new locations (Felt, 1925; Stevens et al., 2010).

The aim of the present doctoral thesis was to gain a deeper understanding of which visual cues are required to set the southerly direction in migrating butterflies and which orientation strategies underlay the behavior of non-migrating butterflies. To investigate this, I presented tethered butterflies of both migratory states (migrating and non-migrating) different visual cues for orientation and recorded their flight performance in flight simulators. In two field seasons, the cue hierarchy of migrating animals was explored by manipulating the natural celestial cues while the animals where actively flying in an outdoor setup (chapter 1). Additionally, indoor experiments allowed me to test single visual cues and their combination under very controlled conditions to reveal which of them are sufficient to guide the butterflies southwards (chapter 1). Similar to this, the orientation of non-migrating butterflies with respect to different visual cues, including the position of the sun, landmarks, and a panoramic skyline, was studied in indoor flight simulators (chapter 2&3). This revealed not only that the animals combine multiple cues (chapter 2) but also that depending on the presented stimuli, a different orientation strategy was displayed (chapter 3). This opened up the great opportunity to test the underlaying principles controlling behavioral outputs by presenting an ambiguous scenery with two identical stimuli set in a 180° relationship (chapter 3).

Thereby, I asked whether the butterflies treat both cues as the same or if they can combine them to a visual scene in which a specific heading is encoded by the butterflies' internal compass.

6.1 Spatial orientation cues in monarch butterflies

6.1.1 The position of the sun

During the day, the sun is the most prominent cue in the sky providing directional information for orientation. Previous studies showed the use of its position to maintain a southerly direction in migrating monarch butterflies (Froy et al., 2003; Merlin et al., 2009; Mouritsen and Frost, 2002). Here, I demonstrated that the sun is the animals' main orientation reference during the fall migration and that the butterflies, similar to dung beetles (el Jundi et al., 2015b), interpret a bright, green light spot (simulates sun) as the 'real' sun (Franzke et al., in preparation (chapter 1)). This raises the question of what defines a stimulus as the sun: its intensity and/or the color? While bees use spectral component to distinguish between the antisolar hemisphere and the sun/solar hemisphere (Edrich et al., 1979), tethered butterflies did not change their behavior with respect to a blue instead of a green sun suggesting that they ignore the spectral component and use the total intensity instead to detect the sun (Franzke et al., 2020 (chapter 2)). Using a simulated sun, I showed for the first time that even butterflies in their non-migratory state perform well oriented flights (Franzke et al., (2022) in press (chapter 3); Franzke et al., 2020 (chapter 2)). However, whether the non-migrating animals rank the sun highest in their cue hierarchy needs to be investigated in future experiments setting multiple cues in conflict as performed in mantis shrimps (Patel and Cronin, 2020), dung beetles (Byrne et al., 2003; Dacke et al., 2014) and migrating monarch butterflies (Franzke et al., in preparation (chapter 1)).

Non-migrating butterflies display menotactic behavior flying in arbitrary directions (dispersal) when tested outdoors viewing the sun (Calvert, 2001; Zhu et al., 2009) or indoors with respect to a simulated sun (Franzke et al., (2022) in press (chapter 3)). In contrast to this, migrating butterflies maintain a southerly heading when tested under natural conditions (Franzke et al., in preparation (chapter 1); Mouritsen and Frost,

2002) and switch to arbitrary directions when tested indoors with a simulated sun as the only orientation reference (Franzke et al., in preparation (chapter 1)). What causes this change from migratory behavior to dispersal behavior? One possible explanation is a difference in the animals' sun compass. In migrating butterflies this compass is able to compensate for the sun's movement over the course of the day (Froy et al., 2003; Merlin et al., 2009; Mouritsen and Frost, 2002) by gaining time-of-day information from the antennae (Merlin et al., 2009). Disruption of this system by removing the animals' antennae led to a loss of migratory behavior with the butterflies heading in arbitrary directions (Merlin et al., 2009). Thus, it is possible that for the dispersal behavior of butterflies the sun compass is not required to be time compensated similar to what has been shown to be true for dispersing *Drosophila* (Giraldo et al., 2018). In my experiments, migrating butterflies were only able to set the correct migratory direction when the simulated sun was combined with polarized light (Franzke et al., in preparation (chapter 1)), suggesting that the use of the time-compensated sun compass either requires multiple cues or polarized light. To test this, the same indoor experiments with a simulated sun and polarized light can be repeated with antennae-less butterflies during their fall migration.

6.1.2 The pattern of polarized light

The question of whether monarch butterflies use the angel of polarized light for orientation was not answered yet, as two studies provide contradicting results (Reppert et al., 2004; Stalleicken et al., 2005). While these previous studies were conducted outdoors (Reppert et al., 2004; Stalleicken et al., 2005), I combined outdoor with indoor experiments in which additional external cues were eliminated. I demonstrated that polarized light alone was not resulting in directed flights of migrating butterflies and needs to be combined with additional celestial cues (Franzke et al., in preparation (chapter 1)). This suggests that the use of polarized light is ranked lower than to the position of the sun in the animals' cue hierarchy for the migration. This is in line with other arthropods such as diurnal dung beetles, bees and mantis shrimps (el Jundi et al., 2014a; Patel and Cronin, 2020; von Frisch, 1949)).

Interestingly, when migratory butterflies view the combination of polarized light and celestial gradients, they maintained well oriented flights in arbitrary directions. Only in combination with the sun, the animals headed in the correct southerly direction (Franzke et al., in preparation (chapter 1)). This difference in orientation behavior can again be explained by the time-compensated sun compass required for migratory behavior. This suggests that while the sun and polarized light together are combined with time information in the compass, a combination with celestial gradients instead of the sun is not sufficient for time compensation. Intracellular recordings strengthen this hypothesis, as the position of the sun and the angle of polarized light are encoded in the butterflies' compass while the neurons showed no integration of the spectral gradient (Heinze and Reppert, 2011; Nguyen et al., 2021). To investigate this is more detail, future experiments are required. Unfortunately, I cannot exclude that the butterflies rely on the presence of UV light while ignoring the angle of polarized light in my experiments. To ensure that the animals rely on skylight polarization, control experiments must be performed in which unpolarized rather than polarized UV light of the same intensity is presented in addition to the sun stimulus. If the time compensation requires polarized light, I expect the butterflies to fly in arbitrary directions under this condition.

6.1.3 Celestial gradients

The spectral contrast of the sky is characterized by a higher amount of longer wavelength (green) light in the solar hemisphere and a relatively higher amount of short wavelength (UV) light in the anti-solar hemisphere (Coemans et al., 1994; el Jundi et al., 2015a). To investigate whether monarch butterflies rely on such a gradient, the same experiments as in el Jundi et al. (2015a) were performed. Briefly, a UV and a green light spot of the same intensity were set 180° apart from each other and presented together before withholding one of them. Here, I found that the butterflies preferred the hemisphere containing the UV light and change their headings by 180° after turning off this light spot (Franzke et al., in preparation (chapter 1)). This strengthen my hypothesis that the spectral contrast is not required for the time-compensated sun compass. Furthermore, in dung beetles, this behavior was only observed when a bright and a dim light spot were presented, generating an intensity gradient without spectral contrast and withholding the bright light source (el Jundi et al., 2015a). Thus, I hypothesize that the butterflies in my experiment used intensity information and might be more sensitive to UV light, so it appears brighter. Such a difference in the animals' sensitivity can, for example, be based on a different number of photo receptors or physiological adaptations increasing the sensitivity of the receptors themselves. The first explanation does not apply to monarch butterflies, as six out of eight photoreceptors of the main retina are green-sensitive while UV-sensitive cells were found less frequently (Sauman et al., 2005; Stalleicken et al., 2006). To test the sensitivity of the different photoreceptors, recordings from the butterflies' eyes could be performed. The knowledge about the sensitivity of the butterflies to light of different wavelength would provide the great opportunity to adjust the intensity of LEDs to the receptor level and study the use of spectral cues at that brightness. Nevertheless, the experiments performed here indicated that monarch butterflies use the total intensity provided by spectral cues to achieve oriented flights (Franzke et al., in preparation (chapter 1)).

6.1.4 Landmarks and the panoramic skyline

Based on the observation that migratory butterflies change their heading direction when flying near the Sierra Madre Oriental (Calvert, 2001), I investigated the use of local cues, landmarks and a panoramic skyline for orientation in monarch butterflies (Franzke et al., (2022) in press (chapter 3); Franzke et al., 2020 (chapter 2)). The butterflies tested here did not maintain a directed course with respect to a simulated panoramic skyline (Franzke et al., 2020 (chapter 2)). This can result from various facts. First, the butterflies tested in this study were in their non-migratory state and, therefore, might do not rely on such a local cue. Thus, the role of a panoramic skyline for the butterflies' migration needs to be investigated during the fall migration. Second, the panoramic skyline and landmark simulated in the experiments were unfamiliar to the butterflies and most likely not linked to an butterflies' goal. While flies associate a dark stripe with a landing site (Maimon et al., 2008), the butterflies seem not to treat such a landmark the same way (Franzke et al., (2022) in press (chapter 3)). However, to gain directional information from landmarks, insects need to associate local cues with their goal (Brünnert et al., 1994; Fleischmann et al., 2016) for example by performing learning walks/flights around their nest entrance (Fleischmann et al., 2018a; Zeil et al., 1996). To answer the question if butterflies can learn local cues for spatial orientation, experiments need to be performed in which the animals can associate a landmark with a reinforcement similar to experiments in flies and ants (Buehlmann et al., 2020; Ofstad et al., 2011). Another possibility is, that the butterflies reaching the Sierra Madre Oriental do not actively adjust their headings by global cues, but rather passively change their direction being funneled by this geographical barrier (Reppert et al., 2010).

Nevertheless, in my experiments the butterflies were not disoriented when perceiving local cues and stabilized their flights for short sequences using optic-flow information (Franzke et al., (2022) in press (chapter 3); Franzke et al., 2020 (chapter 2)). Furthermore, I demonstrated that the internal compass is likely not involved in this orientation strategy (Franzke et al., (2022) in press (chapter 3)). However, this seems to be different when a panoramic skyline was presented in combination with a simulated sun. Here, the animals' directedness was increased (Franzke et al., 2020 (chapter 2)), suggesting that optic flow can either additionally stabilize flights or contributes the butterflies' sun compass. Although it is not fully understood how these multiple cues are integrated in the butterflies' brain, it is most likely that optic-flow information is partly send to the central complex similar to what has been shown in locusts and bees (Rosner et al., 2019; Stone et al., 2017). There, this information is potently integrated together with directional input from the sun contributing to the heading network similar to idiothetic cues (Beetz et al., 2021).

6.2 The brain underlying orientation

The results presented in this doctoral thesis demonstrated how complex the underlying principles of orientation are. Depending on the available stimulus and the butterflies' state (migrating or non-migrating), different orientation strategies are displayed. This must be flexibly controlled by the animals' brain, enabling them to switch between behavioral strategies and rapidly adapt to new environmental factors. A recent study investigating the heading network of monarch butterflies revealed that the compass representation was only operating when the animals were actively flying (Beetz et al., 2021). This was concluded, since the neuronal activity of the head-direction network in resting animals showed a bimodal response to two sun stimuli set 180° apart, while in flying individuals one explicit response was found (Beetz et al., 2021). When I recorded the orientation behavior of tethered butterflies in a flight simulator with respect to two bright stripes, the animals performed bimodal flights frequently switching between fixating both stripes (Franzke et al., (2022) in press (chapter 3)). In contrast, when two sun stimuli were displayed, the butterflies were able to maintain one heading or return to this heading after a rotation in their yaw axis (Franzke et al., (2022) in press (chapter 3)). This suggest that the butterflies' compass is only involved in controlling menotactic and not attraction behavior similar to what has been shown in *Drosophila* (Giraldo et al., 2018). Which pathway is responsible for fixation behavior than? In flying *Drosophila*, motion-sensitive neurons in the lobula plate of the optic lobes were found to be required for robust stripe fixation and based on a model, these neurons are directly connected to descending neurons not involving additional pathways (Fenk et al., 2014). However, whether this applies for monarch butterflies needs to be further tested in anatomical and physiological studies.

In several experiments of this thesis, the butterflies combined different celestial cues. For such combinations, insects may possess an internal prediction of how the cues are correlated, called a matched filter (Wehner, 1987). In locusts, for example, the neuronal responses to polarized light and a simulated sun reflected the natural 90° relationship between both cues, suggesting an innate representation about the constellation of these cues (Pegel et al., 2018). However, no internal representation of the perpendicular relationship of the sun and the main angle of polarized light was found in the monarch butterflies' central complex (Heinze and Reppert, 2011; Nguyen et al., 2021). Another method linking cues is to take a snapshot of the current arrangement and to compare the memorized picture to the current view (Dacke and el Jundi, 2018; el Jundi et al., 2016). Until now, it is not fully understood which strategy (matched filter or snapshot) monarch butterflies use. To behaviorally investigate this, the same combination experiment with a polarizer and a simulated sun as in Franzke et al. (in preparation (chapter 1)) could be performed, but this time with the axis of the linear polarizer in conflict to the position of the sun. If the animals are able to set the correct migratory direction under this condition, they do not possess a matched filter.

6.3 Open questions

The present doctoral thesis focused on the use of visual cues as orientation reference in monarch butterflies. Nevertheless, directional information is not restricted to vision. Some insect including ants and moths (Dreyer et al., 2018a; Fleischmann et al., 2018b) use the Earth's magnetic field for orientation. In monarch butterflies, light-dependent magnetosensory organs were detected, which sense the inclination of the Earth's magnetic field (Wan et al., 2021) and potentially help migrating butterflies to maintain their southerly direction (Guerra et al., 2014). As this cue requires light in a range of 380 to 420 nm (Guerra et al., 2014; Wan et al., 2021) and the LEDs used in all indoor experiments of this thesis have a narrow emission spectrum either in the UV (365 nm), blue (458 nm), or green (520 nm) range, no statement about the use of a magnetic compass can be made here. Thus, to investigate the interaction of a magnetic compass and the sun compass, modifications on the flight simulator must be performed.

Another possible cue that was not investigated in my thesis is the direction of the wind. This is a promising cue as dung beetles were even found to transfer information between the sun- and the wind-compass (Dacke et al., 2019). Especially during migration, when long distances are be traveled, the alignment with favorable winds can be an efficient strategy to reach the overwintering area rapidly.

One question that still needs to be answered is how the butterflies pinpoint their specific overwintering area after reaching Central Mexico. It is hypothesized that odor cues, either of the oyamel trees (Reppert et al., 2010) or the monarch butterflies themselves, inform the animals about their arrival. This could be investigated in flight simulators tethering migratory butterflies and presenting different odors to the animals. Furthermore, in non-migrating butterflies testing the use of *Asclepias* scent can answer the question whether the butterflies rely on olfactory cues to detect their host plants.

The present doctoral thesis provides important insights into how migratory and non-migratory monarch butterflies use and combine different visual cues for orientation. Furthermore, it was demonstrated, that the animals display different orientation strategies and flexibly switch between them. To gain a deeper understanding of which neuronal circuits control these behaviors and which additional cues the butterflies rely on, future behavioral investigations need to be performed in combination with electrophysiological and neuroanatomical experiments.

Abbreviations

CBL	lower unit of the central body
CBU	upper unit of the central body
CX	central complex
DRA	dorsal rim area
NO	noduli
OL	optic lobe
PB	protocerebral bridge

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Franzke, M., Kraus, C., Gayler, M., Dreyer, D., Pfeiffer, K. and el Jundi, B. (2022) in press. Stimulus-dependent orientation strategies in monarch butterflies. Journal of Experimental Biology 2022, jeb.243687.

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Poster presentations

Franzke M., Nguyen T.A.T., Merlin C., el Jundi B.
The combination of polarized light and the position of the sun guides monarch butterflies to Mexico
14. Göttingen Meeting of the German Neuroscience Society

Franzke M., Dreyer D., Warrant E. J., el Jundi B.
The use of spectral cues for orientation in the monarch butterfly *Danaus plexippus*
13. Göttingen Meeting of the German Neuroscience Society

Franzke M., Dreyer D., Warrant E. J., el Jundi B.
The use of spectral cues for orientation in the monarch butterfly *Danaus plexippus*
111. Annual meeting of the German Zoological Society, Greifswald, Germany

Franzke M., Dreyer D., Warrant E. J., el Jundi B.
The use of spectral cues for orientation in the monarch butterfly *Danaus plexippus*
5. Meeting of the Arthropod Neuroscience Network (ANN)

Franzke M., Dreyer D., Warrant E. J., el Jundi B.
The use of spectral cues for orientation in the monarch butterfly *Danaus plexippus*
The international Congress of Neuroethology, Brisbane, Australia

Franzke M., Schmitt F., Rössler W.
Locomotor activity and phototaxis are influenced by the neuropeptides allatostatin A and allatotropin and by light exposure in the dessert ant *Cataglyphis noda*
12. Göttingen Meeting of the German Neuroscience Society

Oral presentations

Franzke M., Dreyer D., Warrant E. J., el Jundi B.
The use of spectral cues for orientation in the monarch butterfly *Danaus plexippus*
30. Neurobiology Doctoral Students Workshop, Würzburg, Germany

Franzke M., Dreyer D., Warrant E. J., el Jundi B.
The use of spectral cues for orientation in the monarch butterfly *Danaus plexippus*
29. Neurobiology Doctoral Students Workshop, Berlin, Germany