The Function of Learning Walks of *Cataglyphis* Ants: Behavioral and Neuronal Analyses

Die Funktion der Lernläufe in *Cataglyphis* Ameisen: eine Studie des Verhaltens und der neuronalen Auswirkungen

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„[…] animal navigators, have used and use no more than the five senses of conventional literature and philosophy, namely, sight, hearing, smell, taste and touch. If they use a sixth sense which might be different or at least partly independent from any of the conventional five, then that sense is a time sense, an ability to perceive accurately the passage of time which may be dependent on „internal rhythm“ at least partly independent of the observation of the movement of the sun.“

Harold Gatty (1958)

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Summary

Humans and animals alike use the sun, the moon, and the stars to guide their ways. However, the position of celestial cues changes depending on daytime, season, and place on earth. To use these celestial cues for reliable navigation, the rotation of the sky has to be compensated. While humans invented complicated mechanisms like the Antikythera mechanism to keep track of celestial movements, animals can only rely on their brains. The desert ant *Cataglyphis* is a prime example of an animal using celestial cues for navigation. Using the sun and the related skylight polarization pattern as a compass, and a step integrator for distance measurements, it can determine a vector always pointing homewards. This mechanism is called path integration. Since the sun's position and, therefore, also the polarization pattern changes throughout the day, *Cataglyphis* have to correct this movement. If they did not compensate for time, the ants' compass would direct them in different directions in the morning and the evening. Thus, the ants have to learn the solar ephemeris before their far-reaching foraging trips.

To do so, *Cataglyphis* ants perform a well-structured learning-walk behavior during the transition phase from indoor worker to outdoor forager. While walking in small loops around the nest entrance, the ants repeatedly stop their forward movements to perform turns. These can be small walked circles (voltes) or tight turns about the ants' body axes (pirouettes). During pirouettes, the ants gaze back to their nest entrance during stopping phases. These look backs provide a behavioral read-out for the state of the path integrator. The ants "tell" the observer where they think their nest is, by looking back to it. Pirouettes are only performed by *Cataglyphis* ants inhabiting an environment with a prominent visual panorama. This indicates, that pirouettes are performed to learn the visual panorama. Voltes, on the other hand, might be used for calibrating the celestial compass of the ants.

In my doctoral thesis, I employed a wide range of state-of-the-art techniques from different disciplines in biology to gain a deeper understanding of how navigational information is acquired, memorized, used, and calibrated during the transition phase from interior worker to outdoor forager. I could show, that celestial orientation cues that provide the main compass during foraging, do not guide the ants during the look-back behavior of initial learning walks. Instead *Cataglyphis nodus* relies on the earth's

magnetic field as a compass during this early learning phase. While not guiding the ants during their first walks outside of the nest, excluding the ants from perceiving the natural polarization pattern of the skylight has significant consequences on learning-related plasticity in the ants' brain. Only if the ants are able to perform their learning-walk behavior under a skylight polarization pattern that changes throughout the day, plastic neuronal changes in high-order integration centers are induced. Especially the mushroom body collar, a center for learning and memory, and the central complex, a center for orientation and motor control, showed an increase in volume after learning walks. This underlines the importance of learning walks for calibrating the celestial compass. The magnetic compass might provide the necessary stable reference system for the ants to calibrate their celestial compass and learn the position of landmark information. In the ant brain, visual information from the polarization-sensitive ocelli converge in tight apposition with neuronal afferents of the mechanosensitive Johnston's organ in the ant's antennae. This makes the ants' antennae an interesting candidate for studying the sensory bases of compass calibration in *Cataglyphis* ants. The brain of the desert navigators is well adapted to successfully accomplish their navigational needs. Females (gynes and workers) have voluminous mushroom bodies, and the synaptic complexity to store large amount of view-based navigational information, which they acquire during initial learning walks. The male *Cataglyphis* brain is better suited for innate behaviors that support finding a mate.

The results of my thesis show that the well adapted brains of *C. nodus* ants undergoes massive structural changes during leaning walks, dependent on a changing celestial polarization pattern. This underlies the essential role of learning walks in the calibration of orientation systems in desert ants.

Zusammenfassung

Die Gestirne helfen nicht nur uns Menschen uns zurecht zu finden, sondern auch Tiere können Sonne, Mond und Sterne zum Navigieren nutzen. Dabei gilt es aber zu beachten, dass die Himmelskörper ihre Position abhängig von der Tageszeit, den Jahreszeiten und dem Standort auf der Erde verändern. Um anhand von Himmelseigenschaften erfolgreich navigieren zu können, ist es deshalb unerlässlich diese Himmelsrotation zu kennen und für diese zu kompensieren. Menschen haben dafür bereits in der Antike komplizierte Maschinen wie den Antikythera Mechanismus entwickelt, Tiere dagegen brauchen nur ihr Gehirn. Wüstenameisen der Gattung *Cataglyphis* sind kleine Meisternavigatoren. Sie benutzen einen Himmelskompass, basierend auf dem Sonnenstand und dem mit ihr assoziierten Polarisationsmuster des Himmels, und einen Schrittintegrator, um einen Vektor zu bestimmen, der immer genau zu ihrem Ausgangspunkt zurück zeigt. Dieser Orientierungsmechanismus heißt Wegintegration. Da sich allerdings die Position der Sonne am Himmel und damit auch das Polarisationsmuster des Himmels über den Tag verändern, muss *Cataglyphis* für diese Veränderung kompensieren. Würde sie das nicht tun, würde ihr Kompass morgens in eine ganz andere Richtung als abends zeigen. Deshalb müssen Ameisen den Sonnenverlauf erlernen, bevor sie zu ihren weitläufigen Futtersuchläufen aufbrechen. Während des Übergangs von Innendiensttier zu Sammlerin, führt *Cataglyphis* dazu ein strukturiertes Lernlaufverhalten durch. Dabei laufen die Ameisen in kleinen Schlaufen um ihren Nesteingang und stoppen ihre Vorwärtsbewegung mehrmalig, um Drehungen durchzuführen. Diese Drehungen sind entweder kleine gelaufene Kreise (Volten) oder Drehungen um die eigene Achse (Pirouetten). Nur *Cataglyphis* Arten*,* die Gegenden mit einem reichhaltigen visuellen Panorama bewohnen, führen Pirouetten aus bei denen sie zurück zu ihrem Nesteingang schauen. Dies legt nahe, dass während Pirouetten das Panorama gelernt wird. Während Volten wird wohl der Himmelskompass kalibriert. Die Rückdrehungen während ihrer Lernläufe geben die einmalige Möglichkeit, die Ameise zu „fragen“ wo sie denkt, dass ihr Nest sei und damit ihren Wegintegrator auszulesen.

In meiner Doktorarbeit kombinierte ich viele biologischen Methoden unterschiedlicher Disziplinen, um zu untersuchen wie die Ameisen ihre Navigationssysteme während der ersten Läufe außerhalb des Nestes erlernen, speichern, kalibrieren und später nutzen. Ich konnte zeigen, dass Himmelsinformationen, die bei Sammlerinnen als

wichtigster Kompass dienen, nicht für die Orientierung der Rückblicke während Lernläufen verwendet werden. Stattdessen nutzten naive *Cataglyphis nodus* das Erdmagnetfeld als Kompass. Obwohl Himmelsinformationen nicht als Kompass während der Lernläufe genutzt werden, spielen sie eine essentielle Rolle für neuroplastische Veränderungen im Gehirn der Ameisen. Nur wenn Ameisen ihre Lernläufe unter einem Polaristaionsmuster, das sich über den Tag hinweg verändert, ausführen, kommt es zu plastischen Veränderungen in neuronalen Integrationszentren. Besonders die Pilzkörper, Zentren für Lernen und Gedächtnis, und der Zentralkomplex, Zentrum für Orientierung und Bewegungssteuerung, nehmen im Volumen nach Lernläufen zu. Lernläufe spielen also eine wichtige Rolle für die Kalibrierung der Navigationsinformationen. Das Erdmagnetfeld könnte das für die Kalibierung notwendige erdgebundene, stabile Referenzsystem bieten, an dem die Himmelsbewegung gelernt wird. Im Ameisengehirn laufen visuelle Informationen von den polarisatiossensitiven Ocelli mit Afferenzen des mechanosensitiven Johnstonschen Organ aus der Antenne zusammen. Die Antenne könnte daher eine wichtiges Organ für die Kalibrierung der Orientierungssysteme sein. Das kleine Gehirn der Ameisen ist bestens an ihre Anforderungen als große Navigatoren angepasst. Weibliche *C. nodus* (Arbeiterinnen und Königinnen) besitzen große Pilzkörper mit einer Anzahl an Synapsen, die es ihnen erlaubt eine Vielzahl von Umgebungsbildern zu speichern, die sie während ihrer initialen Lernläufe lernen müssen. Das männliche *Cataglyphis*-Gehirn ist besser auf angeborene Orientierungsstrategien angepasst, die ihm helfen einen Geschlechtspartner zu finden.

Die Ergebnisse meiner Doktorarbeit zeigen, dass das an die navigatorischen Herausforderungen angepasste Gehirn von *C. nodus* signifikante neuronale Veränderungen in Abhängigkeit eines sich veränderten Polaristaionsmusters während der Lernläufe erfährt. Dies zeigt die essentielle Rolle der Lernläufe in der Kalibrierung der Navigationssysteme von Wüstenameisen.

1 General Introduction

1.1 Animal Orientation and Navigation

Finding our way is quite a demanding task. Whether we aim to locate a room, reach a restaurant arrangement, or drive hundreds of kilometers for vacation, for navigating efficiently, it is essential to know where we are and where we want to go. The same is true for animals. Many animal species must find their way back to their nest, migrate to a specific destination (sometimes over mind-boggling distances), or make their ways in a featureless open ocean. To do so, animals can use a wide variety of orientation strategies involving a multitude of sensory cues.

Such spatial orientation behaviors, defined as any directed behavior, e.g., forward movements or alignments (Grob et al., 2021a), comprise several strategies based on different types of information. In order to distinguish between different orientation strategies, it is helpful to define the information it is based on. To perform directed behavior, an animal at least needs directional information. This directional information can be based on local directional information, which does not provide absolute compass directions (non-compass orientation). Non-compass orientation includes behaviors like taxis, menotaxis, topographic features, idiothetic cues, beaconing, or the use of pheromone trails (Grob et al., 2021a).

In contrast, global directional information provides absolute cardinal directions (compass orientation). With this, animals can determine directions independent from local cues. The best example for a global directional cue and the most familiar one is a magnetic compass. Like with a hand-held magnetic compass, animals can use the earth's magnetic field to determine absolute directions on a global scale (Mouritsen, 2015; Phillips, 1996). In addition, celestial cues, like the sun, the stars, or the moon, can provide compass information. In contrast to the stable magnetic field of the earth, celestial cues change their position in the sky over time. If the cues are to be used as compass cues, the animal must correct for this movement. Otherwise, it would unknowingly change its direction depending on the time of the day, the season, or its place on earth (Kramer, 1950; Pomozi et al., 2001; v. Frisch, 1950; Wiltschko and Wiltschko, 1990). I will discuss this calibration in more detail in the next chapter.

These orientation strategies only provide directional information but lack information about the location, e.g., direction and distance of a goal. A particular case of spatial orientation in which, in addition to directional information also positional information is available to the animal, is navigation (Grob et al., 2021a). The positional information can be either geocentric, i.e., anchored to a geographical position (earth-bound), or egocentric, i.e., fixed to the animal and its position (animal bound). Positional information can be either collected *en route* (egocentric) or is based on information available on site (geocentric). Egocentric navigation includes route following, or path integration, while geocentric navigation uses map information (Able, 2001; Grob et al., 2021a).

1.2 Celestial Compass Calibration

A wide variety of animals species utilize celestial cues for orientation and navigation. In 1950, both v. Frisch (1950) in honey bees and Kramer (1950) in starlings showed for the very first time that animals are indeed capable of using the sun as a compass. As already mentioned, celestial cues come with a big caveat: they change their positions in the sky over time, making them unreliable to use over extended periods of time. Furthermore, this change in position is not linear, and its function is both season- and place-specific. Thus, it cannot be inherently known to the animal (Fleischmann, 2018; Wiltschko and Wiltschko, 1990). However, when an animal learns the movements of the celestial cues and can time compensate for their movements, the sun, the moon, and the stars make a suitable candidate to provide compass directions, i.e., providing global directional information. This is due to their large distance to earth, global availability, and prominence in the sky. Thus, many animals spend an impressive amount of time calibrating their celestial compass systems.

1.2.1 Birds

Birds are some of the best-studied experimental models for orientation and navigation. Migratory birds, for example, cover enormous distances between their summer and winter sides, and the navigational capabilities of carrier pigeons have been exploited by humans for centuries. Birds use a wide variety of navigational strategies to guide them on their long journeys (Pakhomov and Chernetsov, 2020). They use celestial compasses based on the sun, the skylight polarization patterns, and the star pattern and can time compensate for the movement of e.g. the sun (Wiltschko, 1980). In order to use the sun as a reliable compass, birds learn the course of the sun (solar ephemeris) during an early sensitive phase at the beginning of their life. Birds like pigeons have to be able to view the position of the sun at different times of the day (Wiltschko and Wiltschko, 1990). Early learning flights encourage the calibration of the celestial compass in pigeons (Wiltschko and Wiltschko, 1981). Birds need a stable, earthbound reference to calibrate the solar ephemeris. Pigeons, for example, use their magnetic compass as such a reference system (Wiltschko et al., 1998; Wiltschko and Wiltschko, 1990). This calibration against the magnetic field allows birds to use a time-compensated sun compass throughout the day with high accuracy (Wiltschko and Wiltschko, 1990).

1.2.2 Butterflies

Like migratory birds, some butterflies and moths migrate over thousands of kilometers (Reppert and Roode, 2018; Warrant et al., 2016). A prime example of a long-distance migrator in insects is the monarch butterfly (*Danaus plexippus*). During the fall migration, the butterflies travel thousands of kilometers from southeastern Canada and the eastern United States of America to their overwintering sites in central Mexico (Reppert and Roode, 2018). To find their way, they employ an impressive set of orientation cues. The primary cue that steers monarch butterflies during their migration is the sun. They are able to time compensate for the sun's movement and, thus, use the sun as a reliable compass throughout the day (Perez et al., 1997). The neuronal substrate of the butterfly's sun compass is located in the central complex (Heinze et al., 2013; Heinze and Reppert, 2012). Single central complex neurons can integrate both the azimuthal position and the angle of the *E*-vector of the polarized sunlight. This integration is able to represent skylight cues, like the position of the sun and its polarization pattern, and their changing relationship relative to each other throughout the day (Heinze and Reppert, 2011). In order to adequately compensate for these changes over time, the internal clock of the butterflies plays a crucial role in sun compass orientation. Disrupting the internal clock also disrupts time-compensated flight orientation (Froy et al., 2003; Guerra et al., 2012; Merlin et al., 2009). In monarch butterflies, the internal clock for time-compensated orientation is located in the antennae (Merlin et al., 2009). From the antennae, the clock information has somehow to be integrated into the butterfly’s sun compass system. Thus, a theoretical connection between the butterfly’s antennae and the central complex has been proposed (Merlin et al., 2009; Reppert and Roode, 2018). Additionally to the chronometer, the butterfly antennae also house a light-sensitive magneto sensor, which could be used in cooperation with the internal clock to calibrate the monarch butterfly’s sun compass (Guerra et al., 2014).

1.2.3 Hymenoptera

Orientation by a celestial compass is not only used for long-distance travel but also for shorter trips. Honey bees were among the first animals to have been shown to use a time-compensated sun compass orientation (v. Frisch, 1950). Central place foragers such as many bees, wasps, and ants face the crucial challenge of finding their way back to their nest every time they go out and forage. Social Hymenoptera, like honey bees, or ants, that show a division of labor, often spend a significant amount of life in the darkness of their nest. Before switching to being an outdoor forager, the animals calibrate their compass systems and learn their surroundings. This transition phase provides the perfect opportunity to study the calibration of compass systems. During this transition, they perform learning flights, or as the case may be, walks (sometimes also called exploration or orientation flights/walks). During this highly structured early learning behavior, the animals usually turn back to look at a goal, for example, the nest entrance or a feeder. This behavior has been studied intensively in bees (Degen et al., 2015; Lehrer, 1993; Zeil et al., 1996) and wasps (Stürzl et al., 2016; Zeil et al., 1996), as well as ants (Freas et al., 2019; Zeil and Fleischmann, 2019).

During learning flights bees and wasps fly in a series of arcs, increasing in height and radius over time while fixating the nest entrance with their lateral retina. Every time the nest crosses the frontal visual field of the animals, bees and wasps change their flight direction and start a new arc (Degen et al., 2015; Lehrer, 1993; Zeil et al., 1996). This leaves the frontal visual field, the area of the retina with the highest resolution and with the binocular visual field, free to scan the environment. Every new learning flight explores a new sector (Degen et al., 2015). These learning flights are used for learning and memorizing the surroundings of the hive, as well as the landmark panorama

(Capaldi and Dyer, 1999), and calibrating the solar ephemeris (Dyer and Dickinson, 1994, 1996). Bees are able to learn the movement of the sun in relation to the panoramic scenery around their hive (Towne, 2008; Towne and Kirchner, 1998). The panorama provides a stable local reference to calibrate the rotation of the sky as the geomagnetic field does for birds.

The learning walks of ants show many similarities to the learning flight of wasps and bees (Collett and Zeil, 2018). As in bees, learning walks of ants increase in size, and subsequent learning walks explore new sectors (Zeil and Fleischmann, 2019). Ants look back to their goal (nest entrance), too. However, since worker ants cannot fly and are thus not able to move sideways along their nest, they cannot fixate the goal with their lateral retina (Nicholson et al., 1999). To still be able to gaze back, they repeatedly perform stereotypic turns (Freas et al., 2019; Zeil and Fleischmann, 2019). In the desert ant, *Cataglyphis,* these turns are interrupted by short stopping phases directed towards the nest entrance (Figure 1), an inconspicuous hole in the ground (Fleischmann et al., 2017; Wehner et al., 2004). Since the nest entrance is invisible from the ant’s perspective, the nest-directed back-turns have to be guided by a reference system. It has been suggested that this system could be the current state of their path integrator (Müller and Wehner, 2010).

1.3 *Cataglyphis* Desert Ants - An Experimental Model for Navigation

1.3.1 *Cataglyphis’* Navigational Toolkit

Desert ants of the species *Cataglyphis* (Hymenoptera: Formicidae; Foerster 1850) are a well-established experimental model due to their exceptional navigational performances (Ronacher, 2008; Wehner, 2008, 2020). They are faced with the crucial challenge of finding their way back home in their often very hostile habitats. To do so in the shortest way possible, the ants employ a vast variety of navigational strategies, utilizing a multiple of sensory modalities (Wehner, 2020).

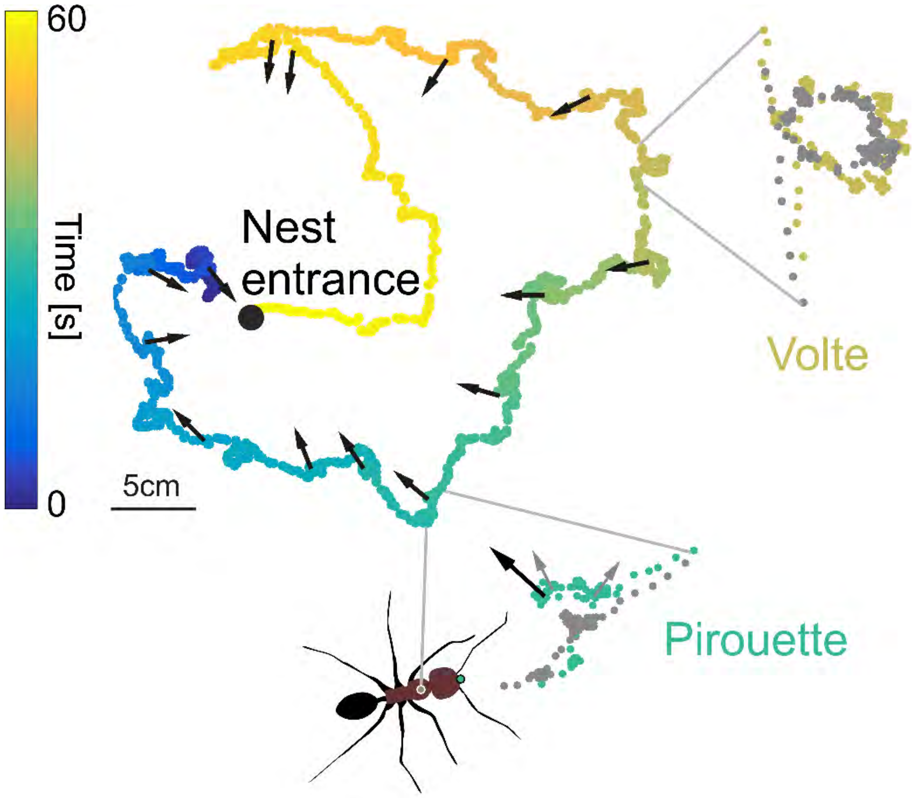


Figure 1 Fine structure of a *C. nodus* learning walk. During a learning walk the ants circle the nest entrance (black dot), including several pirouettes, during which the ant looks back to the nest entrance (black arrows), and voltes, without stopping phases. Pirouettes are tight turns about the ant's body axes. The ants stop multiple times for more than 100 ms (gray arrows), with the longest stop directed towards the nest (black arrow). During voltes, the ants leave their forward path, walk a small circle and return to their original walking direction. The color of the path encodes the time. (Figure adapted from Fleischmann et al., 2020a).

Cataglyphis ants use very different environmental cues to help them find their ways. These include visual cues, such as landmarks, and the panorama (Collett, 2010; Collett et al., 1992; Fleischmann et al., 2016, 2018b; Ronacher and Wehner, 1995; Wehner et al., 1996; Ziegler and Wehner, 1997), olfactory cues, like olfactory landmarks or the smell of food (Buehlmann et al., 2014; Huber and Knaden, 2018; Steck et al., 2009), tactile cues (Seidl, 2006), gravitational information (Grah et al., 2005; Wohlgemuth et al., 2001), wind directions (Müller and Wehner, 2007), tactile cues (Seidl, 2006), as well as vibrational and magnetic landmarks (Buehlmann et al., 2012). These orientation cues can be learned and memorized by ants, e.g. to be used for route navigation (Collett, 2010; Grob et al., 2021a; Kamhi et al., 2020; Wehner, 2020; Wehner et al., 2006).

Cataglyphis' primary navigational strategy is path integration (sometimes also called "vector navigation" or "dead reckoning"). In order to find the fastest and shortest way back to their colony, the ants integrate information about their distance traveled, and the directions walked into a vector pointing homeward (home vector) (Müller and Wehner, 1988). To determine directions traveled, *Cataglyphis* uses a celestial compass, mainly based on the sun's position and the polarization pattern of the sky (Wehner, 1989; Wehner and Müller, 2006). These celestial compass cues are of higher importance for the ant's navigation than the less reliable idiothetic cues (Lebhardt et al., 2012). However, as mentioned above, since the celestial cues are changing their position throughout the day, these movements have to be learned and calibrated in order to serve as reliable compass cues (Wehner and Müller, 1993). This is especially crucial for *Cataglyphis'* foraging trips, which usually take place during the hottest times of the day, i.e., when the sun changes position the fastest (Fleischmann, 2018; Wehner, 2020).

In order to determine the length of the home vector, i.e., the distance traveled, *Cataglyphis* uses an odometer that integrates their steps (Wittlinger et al., 2006, 2007a). Additionally, the ants can also use optic flow for measuring traveled distances (Pfeffer and Wittlinger, 2016; Ronacher and Wehner, 1995). The path integrator is updated continuously during their far-reaching foraging trips, which the ants often perform at high walking speeds (Pfeffer et al., 2019). When an ant finds a food item or decides for other reasons to return to the nest, it switches from a random search behavior to following the home vector. Thus, they find the most direct and fastest way back home (Ronacher, 2008; Wehner, 2020). After returning to the nest, the home vector is reset to zero (Knaden and Wehner, 2006).

Path integration is an especially beneficial navigational strategy in featureless habitats, like North African saltpans, or in (yet) unknown environments since it does not rely on local learned information (Grob et al., 2021a).

1.3.2 Learning Walks of *Cataglyphis*

However, before *Cataglyphis* can become successful foragers, finding their way through the burning desert heat, they have to calibrate their compass systems and learn landmark and panoramic cues. Studying the behavior in the context of their natural and ecologically relevant habitat is essential to understand how ants, upon leaving the nest for the first time, so-called "novices", acquire their navigational knowledge.

Cataglyphis workers undergo an age-related polyethism, spending their first four weeks underground inside a dark nest to perform nest-related tasks (Schmid-Hempel and Schmid-Hempel, 1984). Subsequently, the ants leave the darkness of the nest to become outdoor foragers. During their first short walks, novices stay close to the nest entrance and never bring back any food items. Instead, the ants perform stereotyped learning walks around the nest entrance (Fleischmann et al., 2016, 2017; Stieb et al., 2012; Wehner et al., 2004). Each learning walk explores a different sector around the nest entrance. With increasing experience, learning walks lead the ants farther away from the nest entrance until they start to forage (Fleischmann et al., 2016, 2017, 2018b). The ants perform this remarkable behavior for 2–3 days (Fleischmann et al., 2016, 2017; Stieb et al., 2012; Wehner et al., 2004). This duration correlates with the time needed to induce structural neuronal changes for the formation of stable long-term memories after associative learning (Falibene et al., 2015; Hourcade et al., 2010; Schmitt et al., 2016; Scholl et al., 2015; Stieb et al., 2010, 2012). During learning walks, *Cataglyphis* ants repeatedly stop their forward movement to perform turns. There are two distinct types of turns performed by *Cataglyphis*: voltes and pirouettes (Figure 1 & 2). During voltes, the ants walk in a small 360°-circle off their walking path before moving on in the former direction (Fleischmann et al., 2017). These turns are similar to those performed by dung beetles perform on top of their dung ball. During these rotations, dung beetles take snapshots of skylight to align their celestial compass system (el Jundi et al., 2016). Desert ants may likewise use voltes to systematically calibrate their celestial compass system over the day (Fleischmann et al., 2017).

During pirouettes, on the other hand, the ants perform tight turns about their body axes. These pirouettes can be either full (360°) or partial turns (<180°) (Fleischmann et al., 2017; Wehner et al., 2004). The ants interrupt their rotational movements multiple times by stops longer than 100 ms. During the longest of these stops, the gaze direction is accurately directed towards the nest entrance (Figure 1), which is invisible from the ants' perspective. To do so, they must use path integration to align their body axes in the direction of the home vector.

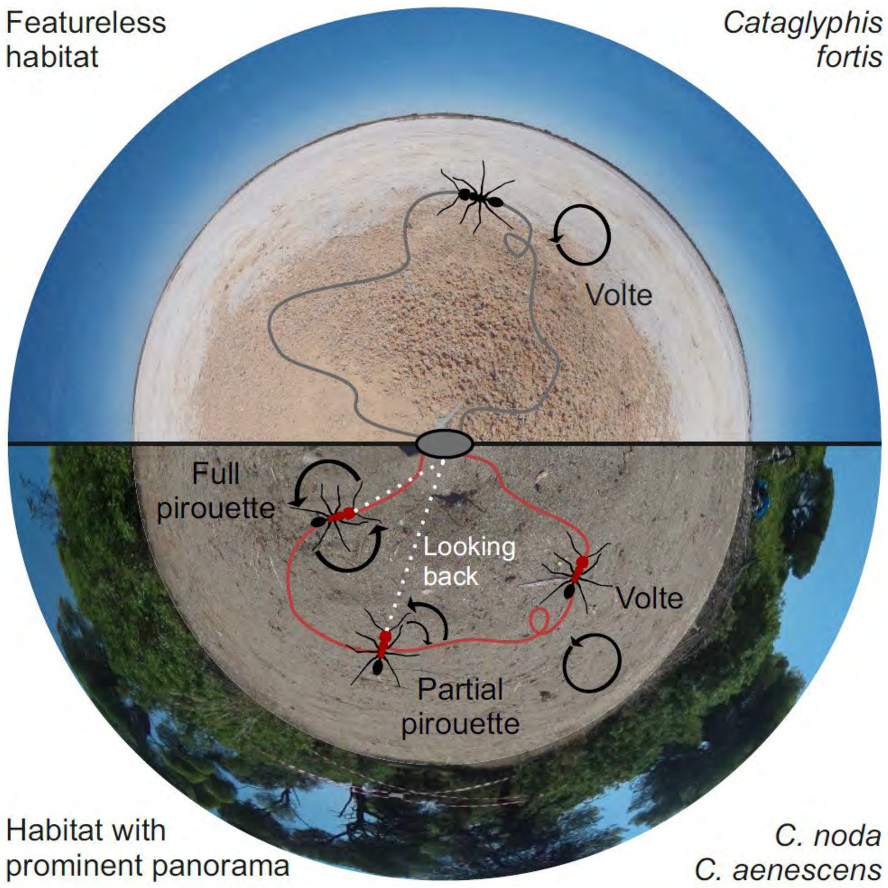


Figure 2 Characteristics of learning walks performed by different *Cataglyphis* species. *C. fortis* that inhabits the featureless saltpans of Tunisia only performs voltes during their learning walks. In contrast, *Cataglyphis* species that inhabit environments with a prominent panorama also perform partial and full pirouettes that include nest-directed stopping phases (indicated by dashed lines). Note that *C. nodus* was formerly sometimes called *C. noda* (see Rössler, 2019 for details). (Figure from Pauline Fleischmann and Robin Grob, first published in Fleischmann (2018)).

By comparing closely related *Cataglyphis* species living in different habitats, it has been shown that only desert ants living in habitats with a prominent panoramic scenery perform pirouettes (Figure 2) (*Cataglyphis nodus* (Note that *C. nodus* was formerly sometimes called *C. noda* (see Rössler, 2019 for details)*, Cataglyphis aenescens* (Fleischmann et al., 2017); *Ocymyrmex robustior* (Müller and Wehner, 2010); *Cataglyphis bicolor* (Wehner et al., 2004); for reviews see Freas et al., 2019; Zeil and Fleischmann, 2019). However, *Cataglyphis fortis* that inhabits featureless salt pans, exclusively performs voltes (Figure 2) (Fleischmann et al., 2017). This indicates that pirouettes and the look-back behavior are related to learning the panoramic scenery or visual landmark cues. Snapshots taken and memorized from different positions around their nest would allow the ants to determine the nest location from the panoramic scenery (Cartwright and Collett, 1983; Fleischmann et al., 2017; Graham et al., 2010; Zeil, 2012; Zeil and Fleischmann, 2019). During learning walks, *Cataglyphis* needs at least two days in order to use panoramic information for navigation (Fleischmann et al., 2016, 2018b). Additionally, the ants need enough space around the nest entrance for performing learning walks in order to memorize useful snapshots for navigation (Fleischmann et al., 2018b).

1.3.3 The *Cataglyphis* Brain

During this early learning phase, the tiny ant brain has to cope with a diverse set of new information and undergoes massive plastic changes (Rössler, 2019). This synergy between learning walk behavior and plastic neuronal changes makes learning walks a perfect subject for studying the interactions between brain, environment, and behavior.

As the neuronal substrate controlling behavior, the brain of ants reflects in the size and structure of the associated brain neuropils the evolutionary needs of the animals (Gronenberg, 2008). The neuropils are subdivisions of the ants' brain that can be ascribed to different functions (Figure 3) (Gronenberg, 2008; Habenstein et al., 2020). Visual information is detected by the compound eyes of the ants and projected into the optic lobes. In each brain hemisphere, one optical lobe is present. It comprises the lamina, the medulla, and the lobula (Habenstein et al., 2020). Visual information plays a crucial role in *Cataglyphis'* navigation strategies. While the whole compound eye detects panoramic and visual information, information about the polarization of the skylight used for the ants' celestial compass is only detected in the dorsal rim area of the compound eye (Labhart and Meyer, 1999; Wehner, 2020). Polarization information is only detected in the UV spectrum (Duelli and Wehner, 1973). The information is then transferred from the optic lobes to the anterior optic tubercle, from which it is projected to the lateral blubs and terminates in the central complex (Figure 3) (Grob et al., 2019; Homberg et al., 2011; Rössler, 2019; Schmitt et al., 2016). Since both distance information (optical flow) and directional information (sky polarization pattern) are integrated in the central complex, it is regarded as the neuronal correlate for path integration (Honkanen et al., 2019; Stone et al., 2017; Webb, 2019).

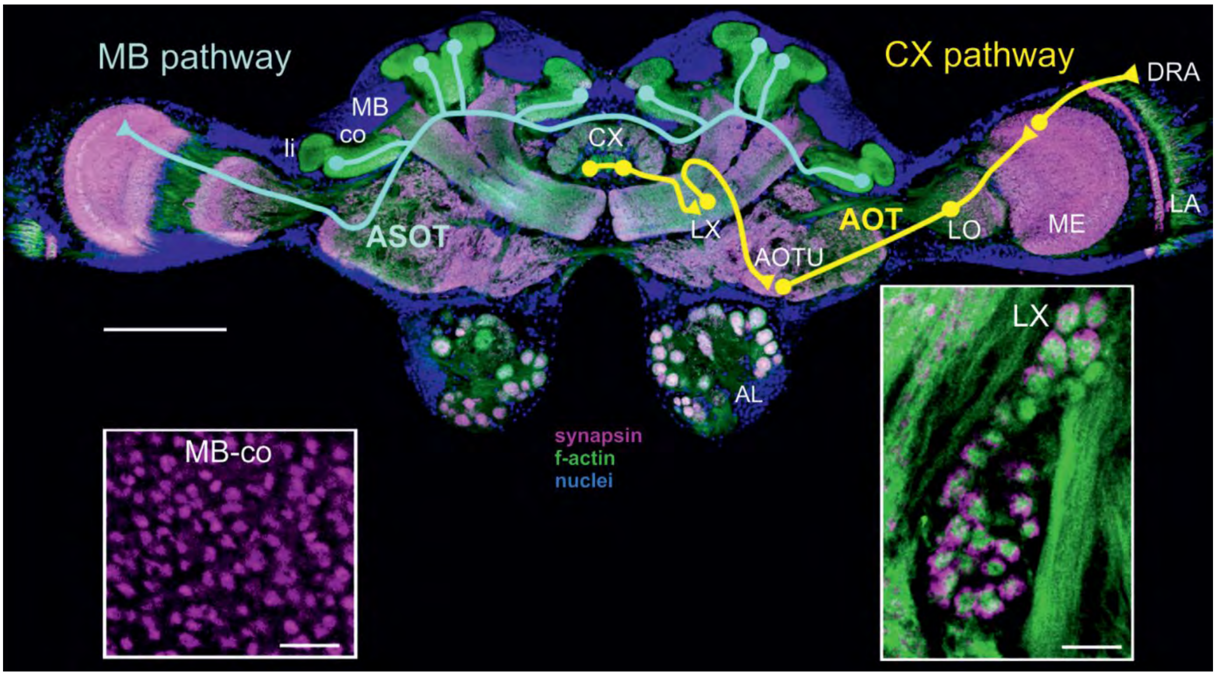


Figure 3: The *Cataglyphis* Brain. Visual information is transferred from the optical lobes to the central brain. The optic lobes comprise lamina (LA), medulla (ME), and lobula (LO). Panoramic visual information is projected via the anterior superior optic tract to the visual input regions of the mushroom bodies (MB) the collar (co). Skylight polarization information is detected in the dorsal rim area of the compound eye (DRA) and transferred via the anterior optical tract (AOT) to the anterior optic tubercle (AOTU), the later complex bulbs (LX) and terminates in the central complex (CX). Olfactory information is projected from the antennal lobes (AL) to the olfactory input region of the MBs, the lip (li). Scale bar = 200 µm. Inlays show the synaptic complexes of the MB-co and the LX. Scale bars = 10µm. The brain is labeled with an antibody to the presynaptic protein synapsin (magenta), staining of f-actin in dendritic and axonal profiles by phalloidin (green), and detection of cell nuclei with Hoechst 3458 (blue). Figure is taken from (Grob et al., 2019).

The central complex is located at the mid-line of the brain. It is an unpaired high-order integration center essential for orientation and navigation (Bender et al., 2010; Dell-Cronin et al., 2021; Grob et al., 2019, 2021a; Honkanen et al., 2019; Pfeiffer and Homberg, 2014; Stone et al., 2017). The central complex integrates a variety of sensory inputs required for orientation and navigation. It is best known for coding celestial compass cues (Homberg et al., 2011; Honkanen et al., 2019; Turner-Evans and Jayaraman, 2016), but its neurons also respond to visual panoramic and mechanosensory information and motion cues. The central complex of the insect brain is also involved in motion control (Martin et al., 2015), landmark memory (Neuser et al., 2008), landmark orientation, and angular path integration (Seelig and Jayaraman, 2015), and is required for spatial working memory during navigation tasks (Dell-Cronin et al., 2021; Neuser et al., 2008; Pisokas et al., 2022). The central complex comprises several neuropils: the central body, the protocerebral bridge, and the paired noduli. The central body can be divided into the upper unit (also called fan-shaped body) and lower unit (also called ellipsoid body) (Habenstein et al., 2020; Ito et al., 2014). The upper unit of the central complex receives input from the lateral complex bulbs (Homberg et al., 2011; Schmitt et al., 2016). The polarization information is then transmitted from the upper unit to the contralateral hemisphere of the protocerebral bridge. The electric field vectors (E-vector) of polarized light are mapped in a topographic representation (Heinze and Homberg, 2007; Heinze and Reppert, 2012; Homberg et al., 2011). Along this visual pathway, the number of synaptic complexes in the lateral bulb of *Cataglyphis* increases after first exposure to the natural skylight (Schmitt et al., 2016). These synaptic complexes are an important relay station in the visual pathway of polarized light, which plays a crucial role in the ants' skylight compass (Habenstein et al., 2020).

In contrast to polarization information, panoramic information is transferred directly from the optical lobes into the mushroom bodies, a higher-order sensory integration center for learning and memory (Figure 3) (Grob et al., 2019; Habenstein et al., 2020; Rössler, 2019). The mushroom bodies are paired neuropils in the dorsal region of the ant brain. They comprise calyces, the pedunculus, the vertical-, and the medial lobe. The calyx, a major sensory input region, is subdivided into an olfactory input region (lip) and a visual input region (collar) in ants (Habenstein et al., 2020; Ito et al., 2014). The mushroom bodies of ants play a crucial role in learning and memorizing olfactory and visual information in the lip and the collar, respectively (Rössler, 2019). As important centers for learning and memory, the mushroom bodies show a substantial degree of neuronal plasticity. The volume of the mushroom bodies and its synaptic complexes (microglomeruli) show plastic changes in relation to the transition phase in *Cataglyphis* (Kühn-Bühlmann and Wehner, 2006; Stieb et al., 2010, 2012). The mushroom bodies also play a crucial role in orientation strategies based on learned information (Buehlmann et al., 2020; Kamhi et al., 2020).

Besides visual information, the brain of *Cataglyphis* has to integrate many other sensory stimuli. The ants' antennae are a crucial sensory organ that detect a wide variety of sensory cues (Hölldobler and Wilson, 1990). Olfactory information is transmitted from the sensory receptor neurons of the antennae to the antennal lobes. The antennal lobes are subdivided into many small glomeruli, the odor processing units. The antennal lobe is the first odor processing center in the ant brain. Olfactory information is then transmitted i.a. to the mushroom body lip (Habenstein et al., 2020; Kirschner et al., 2006; Zube and Rössler, 2008). In addition to olfactory receptors, the antenna also houses gustatory and mechanosensory receptor neurons. The latter plays a crucial role in the reception of wind, vibration, and gravity (Vowles, 1954b; Yack, 2004). Afferents from these non-olfactory receptor neurons project to the antennal mechanosensory and motor center and other sensory input regions of the central adjoining neuropils (Habenstein et al., 2020). *Cataglyphis* utilizes all of the mentioned sensory cues detected by the antennae during orientation and navigation (Wehner, 2020).

1.4 Thesis outline

Cataglyphis desert ants provide a unique opportunity to study their exceptional navigational capabilities in a true neuroethological manner using the whole animal approach. In my doctoral thesis, I investigated how *Cataglyphis nodus* are able to learn the panoramic scenery and the path of the sun throughout the day (solar ephemeris) and, thus, calibrate their compass systems. For this, I focused on the learning-walk behavior during the ants' transition from interior worker to outdoor forager. I studied the interactions between different compass cues and the influence of celestial information on neuroplasticity. During my doctoral project, I was able to combine behavioral field experiments with naturally behaving animals with a wide variety of state-of-the-art neurobiological techniques. In the following chapters of this thesis, I will try to shed some light on the big question of how the tiny brain of the desert ant is capable of learning, calibrating, integrating, and adapting to all the sensory stimuli needed to become a successful forager.

At the beginning of my doctoral thesis, the main question was how *C. nodus* ants were able to look back to their nest entrance as precisely and as accurately as they do (Fleischmann et al., 2017). Since it had previously been suggested for other desert ants that they must use path integration (Müller and Wehner, 2010), we hypothesized that they might use their main compass for path integration during foraging - the celestial compass - to do so. To test this hypothesis, we altered the skylight conditions above the ants' natural nest entrance in the pine forests of Greece and tracked their gaze directions. In addition, we dissected ants that had performed natural learning walks under these altered skylight conditions and stained their brains with antibodies to synaptic proteins to be able to quantify neuroanatomical differences in higher-order integration centers that had occurred due to our experimental conditions (**Chapter 2**: Grob et al. (2017)). Contrary to our hypothesis, not even excluding the ants from their most important celestial cues, the position of the sun and the polarization pattern of the sky (Wehner, 2020), was enough to prevent the ants from looking back (Grob et al., 2017). The only compass left that the ants could potentially use, seemed to be a magnetic compass. Thus, we built an electromagnetic flat coil and installed it around the nest entrance. When powered, this electromagnet disarrayed the earth's magnetic field in a way that it would be of no use as a compass cue. This was the first manipulation that prevented the ants from looking back to their nets entrance while they were still performing learning walks (**Chapter 3**: Fleischmann et al. (2018a)).

The following year, we returned to the Greek pine forest with a more sophisticated electromagnet, a Helmholtz coil. This time, we systematically turned the magnetic field vector around the nest entrance. This allowed us to test our hypothesis, whether the path integrator of *C. nodus* indeed was using compass information based on the earth's magnetic field during learning walks (Fleischmann et al., 2018a). By rotating the horizontal component of the magnetic field, we were able to alter the gaze directions of the ants predictably towards the fictive position of the nest entrance.

With this newly gained knowledge, we asked where the magnetic sensor in the ants might be located. Previous studies had suggested that the insect antennae might be involved in magnetoreception (Abraçado et al., 2008; de Oliveira et al., 2010; Guerra et al., 2014; Lucano et al., 2006; Wajnberg et al., 2010, 2017). The ant antennae is involved in several tasks related to orientation behavior, including olfaction (Hölldobler and Wilson, 1990), wind compass orientation (Müller and Wehner, 2007; Sane et al., 2007), or graviception (Vowles, 1954b). However, the antennae of desert ants had not been studied in great detail. A promising sensory organ that might be involved in both wind compass orientation and graviception, and possibly even magnetoreception, is the Johnston's organ (**Chapter 4**: Grob et al. 2021c)). Using a combination of light microscopy, electron microscopy, and fluorescent retrograde stainings digitized using confocal laser scanning microscopy, we reconstructed the sensory organ and its afferent projections into the ant brain (Grob et al., 2021c). In addition, we compared the Johnston's organ from the ambulatory worker cast with the airworthy reproductive cast, including virgin queens (gynes) and males. Here, we could find small differences between the castes (Grob et al., 2021c).

Since the castes and sexes of ants have very different lifestyles and, thus, also very different navigational challenges, we collected gynes, males, and workers in Greece to compare their neuronal architecture (**Chapter 5**: Grob et al. (2021b)). The most obvious difference is that gynes and males are winged and, therefore, able to fly, while all workers are wingless and purely ambulatory. In addition, their main objectives are quite different. While workers have to perform a variety of tasks, from indoor food storage to outdoor forager, males only need to find a suitable mating partner and mate before they die. Newly mated queens, in contrast, need to find a new colony on their own. We could show that the neuro architecture of the brain reflects the different ecological needs of the castes and sexes. While the male brain is more tuned on innate behaviors, the female brain is well suited for flexible learning-based behaviors (Grob et al., 2021b).

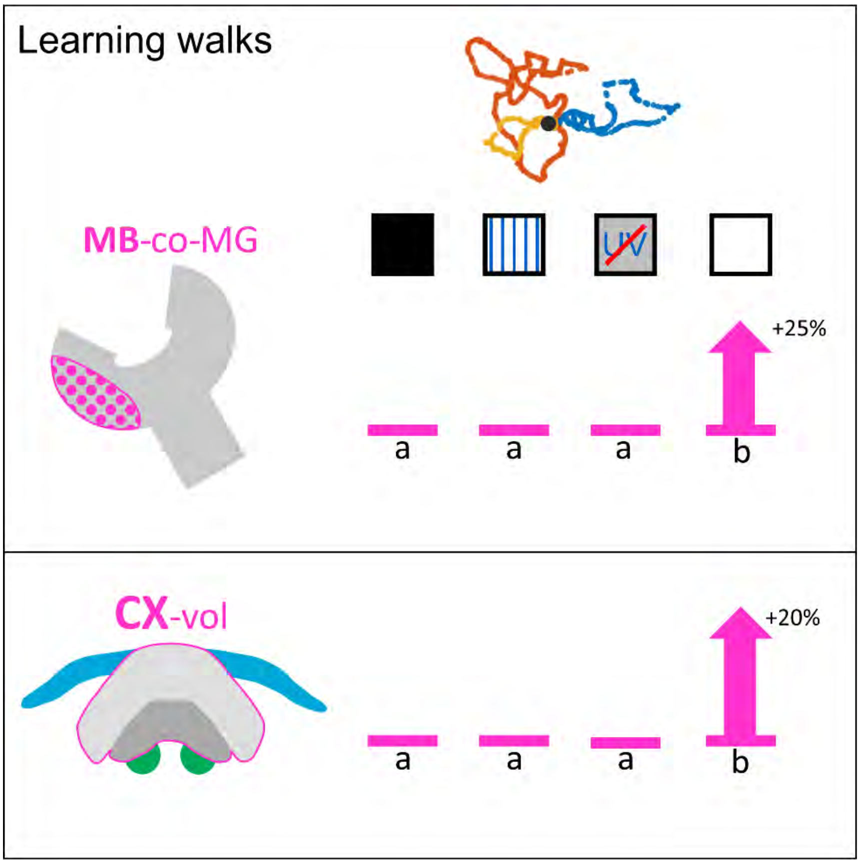
In the last project of this thesis, I want to come full circle and build upon the results I started with. At the beginning of my doctoral project, we could show that the natural skylight, including a moving polarization pattern, is necessary to induce plastic neuronal changes in high-order integration centers for learning and memory, as well as orientation (Grob et al., 2017). We now aimed to investigate what is necessary to trigger this plasticity (**Chapter 6**: Grob et al. (2022)). To test this, we exposed ants that had not yet performed learning walks to different skylight conditions without the possibility to perform learning walks. In addition, we presented ants with a linear polarizer that was rotated throughout the day over the natural nest entrance while the ants could freely perform their learning walks. As a result, we could show that both the performance of learning walks in combination with a changing polarization pattern are necessary to induce neuronal plasticity in higher-order sensory integration centers, while sensory exposure alone leads to synaptic plasticity at upstream relay stations (Grob et al., 2022).

At the end of this thesis, I will discuss the implications of these results for the big questions of how the tiny desert ant brain can set up and calibrate their navigational systems and what these results can tell us about other impressive navigators (**Chapter 7**).

"I don't go far in the beginning, I go some distance and come back again, then in another direction and come back, and then again in another direction. Gradually I know how everything is and can go out far without losing my way."

Quoted in Harold Gatty's 1958 book Nature is your guide: How to find your way on land and sea by observing nature.

2 Manuscript 1: The Role of Celestial Compass Information in *Cataglyphis* Ants during Learning Walks and for Neuroplasticity in the Central Complex and Mushroom Bodies



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The Role of Celestial Compass Information in *Cataglyphis* Ants during Learning Walks and for Neuroplasticity in the Central Complex and Mushroom Bodies

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Central place foragers are faced with the challenge to learn the position of their nest entrance in its surroundings, in order to find their way back home every time they go out to search for food. To acquire navigational information at the beginning of their foraging career, *Cataglyphis noda* performs learning walks during the transition from interior worker to forager. These small loops around the nest entrance are repeatedly interrupted by strikingly accurate back turns during which the ants stop and precisely gaze back to the nest entrance—presumably to learn the landmark panorama of the nest surroundings. However, as at this point the complete navigational toolkit is not yet available, the ants are in need of a reference system for the compass component of the path integrator to align their nest entrance-directed gazes. In order to find this directional reference system, we systematically manipulated the skylight information received by ants during learning walks in their natural habitat, as it has been previously suggested that the celestial compass, as part of the path integrator, might provide such a reference system. High-speed video analyses of distinct learning walk elements revealed that even exclusion from the skylight polarization pattern, UV-light spectrum and the position of the sun did not alter the accuracy of the look back to the nest behavior. We therefore conclude that *C. noda* uses a different reference system to initially align their gaze directions. However, a comparison of neuroanatomical changes in the central complex and the mushroom bodies before and after learning walks revealed that exposure to UV light together with a naturally changing polarization pattern was essential to induce neuroplasticity in these high-order sensory integration centers of the ant brain. This suggests a crucial role of celestial information, in particular a changing polarization pattern, in initially calibrating the celestial compass system.

Keywords: look-back behavior, desert ants, vector navigation, sky-compass pathway, memory, central complex, mushroom body, visual orientation

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INTRODUCTION

Before starting their foraging career central place foragers, like bees, wasps and ants, have to acquire knowledge about the position of their nest in its surroundings and need to calibrate their navigational toolkit (Collett et al., 2013; Fleischmann et al., 2016). In order to do so, they perform learning flights or walks. Studies of this early learning behavior in bees (Opfinger, 1931; Hempel de Ibarra et al., 2009; Philippides et al., 2013; Degen et al., 2015), wasps (Zeil et al., 1996; Stürzl et al., 2016) and ants (Wehner et al., 2004; Fleischmann et al., 2016, 2017) revealed striking parallels in the general sequence of this behavior (Zeil, 2012). When leaving the nest entrance for the first time honeybees (Lehrer, 1993), bumblebees (Hempel de Ibarra et al., 2009; Collett et al., 2013; Philippides et al., 2013) and wasps (Zeil et al., 1996; Stürzl et al., 2016) turn back immediately towards their nest entrance and look back before flying in multiple arcs parallel to the nest entrance. As walking insects do not walk sideways, ants perform repeated turns during their learning walk loops and make stops to look back towards their nest entrance (Wehner et al., 2004; Fleischmann et al., 2017). During these looks back the animals most probably learn the landmark panorama (honeybees: Opfinger, 1931; Lehrer, 1993; bumblebees: Collett et al., 2013; ants: Fleischmann et al., 2016, 2017). Over time the arcs or loops increase in size, and novices move farther away from the nest entrance, while still looking back towards it (Zeil et al., 1996; Wehner et al., 2004; Philippides et al., 2013; Fleischmann et al., 2016). Likewise, experienced foragers perform a learning behavior that includes looks back to the nest, e.g., when experienced animals had difficulties pinpointing their nest (Zeil, 1993; Zeil et al., 1996) or when the nest surrounding had changed drastically (Müller and Wehner, 2010; Narendra and Ramirez-Esquivel, 2017).

However, to determine the direction of the nest entrance from various positions in space, the animals need some kind of reference system. It has been previously proposed, that this system could be part of the path integrator (Graham et al., 2010; Müller and Wehner, 2010), which integrates information about the walked directions (compass) and the distance covered (odometer) into a vector pointing towards the starting point. In *Cataglyphis* ants the path integrator is the main navigational tool (Müller and Wehner, 1988). The ants use an odometer (Wittlinger et al., 2006) and optic flow (Pfeffer and Wittlinger, 2016) to determine the distance covered. By integrating the odometer information with information about the walked directions, for which the ants use the celestial compass (Müller and Wehner, 1988; Wehner et al., 1996; Wehner, 2003), they determine a vector pointing homewards. The celestial compass mainly relies on information about the position of the sun and the skylight polarization pattern in the UV-spectrum (Duelli and Wehner, 1973). This suggests that the skylight polarization pattern only in the UV-spectrum could provide a suitable reference system for the compass information of the path integrator to align gaze directions during learning walks.

The polarization direction of the UV-skylight is detected by specialized ommatidia in the dorsal rim area of the compound eye (Labhart and Meyer, 1999). The information is transferred by neurons forming the anterior optical tract (AOT) via several stages into the central complex (CX; Schmitt et al., 2016). In the CX polarization of the skylight is represented in a map-like pattern (Heinze and Homberg, 2007; Homberg et al., 2011; Heinze and Reppert, 2012). The CX was also shown to be involved in several tasks closely linked to orientation and navigation (Pfeiffer and Homberg, 2014; Fiore et al., 2017). In *Drosophila* the CX is additionally involved in landmark memory (Neuser et al., 2008), landmark orientation and angular path integration (Seelig and Jayaraman, 2015). Another prominent neuronal pathway in bees and ants, the anterior superior optical tract (asot), transfers visual information into the visual subregions of the mushroom bodies (MB; Gronenberg, 2001; Yilmaz et al., 2016). The MBs are centers for sensory integration, learning and memory. They undergo substantial neuronal changes when exposed first time to light (*Drosophila*: Barth and Heisenberg, 1997; *Apis*: Scholl et al., 2014; *Cataglyphis*: Seid and Wehner, 2009; Stieb et al., 2010, 2012) and during the formation of long-term memory (*Acromyrmex*: Falibene et al., 2015; *Apis*: Hourcade et al., 2010).

The duration of learning walk behaviors lasts for up to 3 days (Wehner et al., 2004; Stieb et al., 2012; Fleischmann et al., 2016). This correlates with the time needed for stable long-term memory formation (Menzel, 2001; Hourcade et al., 2010; Falibene et al., 2015; Scholl et al., 2015) and the time needed to induce neuronal changes in the visual subregions of the MBs after exposure to light pulses in *Cataglyphis fortis* (Stieb et al., 2010, 2012). Therefore, learning walks are perfectly suited to study brain-behavior-environment interactions. In this study, we restricted the input into the sky-compass of *Cataglyphis noda* during their early learning walks to ask, which reference system the ants use during this early learning phase to align their gaze directions. Ants that participated in the behavioral field experiments were subsequently used for neuroanatomical analyses. This allowed us to look at the interaction between the learning-walk behavior, the received information during these walks, as well as changes in the neuronal architecture in the terminal stages of two visual pathways, the CX and the MBs. The results suggest that natural skylight polarization information with the UV part of the light spectrum present induce structural changes in the CX and the MBs indicating their role in the initial calibration of visual pathways processing celestial information. However, exclusion of sky-compass information did not prevent *C. noda* from looking back towards their nest entrance suggesting, that celestial cues do not serve as the initial reference system for compass information during learning walks.

MATERIALS AND METHODS

Animals

Experiments were conducted with *C. noda* (Brullé 1832) (**Figure 1A**) in Schinias National Park, Marathonas, Greece from June–August 2016. A colony with a nest entrance in the middle

FIGURE 1 | Experimental setup for skylight manipulation experiments. **(A)** Unmarked *C. noda* ants at the nest entrance. **(B)** 30 cm above the nest entrance, a filter was placed in order to alter the skylight information. Learning walks were recorded with a high-speed 4K-camera. In addition, a HD-camcorder recorded the nest entrance for the whole day. **(C)** Panoramic image of the UV-block with sunshade setup (UVBS). The observer was located in the south to trigger the high-speed recording and to prevent unmarked ants from leaving the area covered by the filter through the opening in the fence, which was located in the south-west.

of a small clearing in the pine forest of the national park (38°08’N 24°01’E) was used for the experiments. In order to make sure that only novices (ants performing learning walks for the first time) were used, all ants leaving the nest were marked on at least three consecutive days before the experiment using car paint (Motip Lackstift Acryl, MOTIP DUPLI GmbH, Haßmersheim, Germany). Unmarked ants can then be considered to be naïve, as it was shown in previous studies (Fleischmann et al., 2016, 2017). The animals were allowed to perform learning walks for three consecutive days within an arena (60 cm × 60 cm) restricted by a transparent plastic fence. Only marked foragers were allowed to leave the restricted area through a small exit in the fence.

Manipulation of the Skylight

To manipulate the skylight the ants perceived during their learning walks, different filter systems (60 cm × 60 cm; **Table 1**) were placed 30 cm above the nest entrance from the third day of marking. Thereby, ants that did not leave the nest, but stayed inside of the nest entrance area would only perceive the altered skylight. The ants could still encounter the landmark panorama in the setup. As a control for the setup, a UV-permeable plexiglass was installed above the arena that did not alter the skylight perceived by the ants (UV100). To alter the skylight polarization pattern to an artificial, fixated one, a linear polarization filter was used. To test whether

TABLE 1 | Groups and filter systems used for skylight manipulation.

Group	Icon	Conditions	Analyses
DD	■	Interior workers that had not yet performed learning walks (excavated in the dark using red light);	Neuroanatomy
UV-Block with sunshade (UVBS)	UV	Three days of learning walks under a UV-light impermeable filter (Plexiglas (Gallery) 0A570 GT, Evonik Performance Materials GmbH, Essen, Germany) blocking 99.7% of the light below 420 nm with a sunshade, to additionally disguise the position of the sun;	Neuroanatomy Gaze direction
Diffusor (Dif)	⧄	Three days of learning walks under a diffusor that lets UV-light pass (Plexiglas (GS) 2458 SC, Evonik Performance Materials GmbH, Essen, Germany), but diffuses any polarization pattern in the skylight;	Neuroanatomy
Polarization filter (P)	▥	Three days of learning walks under a polarization filter (OUV6060-C—HNP’B replacement, Knight Optical Ltd., Harrietsham, United Kingdom) that lets UV-light pass, but provides an artificial linear, fixed polarization pattern;	Neuroanatomy Gaze direction
UV100	□	Three days of learning walks under a UV-light permeable Plexiglas (Plexiglas (GS) 2458, Evonik Performance Materials GmbH, Essen, Germany), as a control for the setup;	Neuroanatomy Gaze direction
No filter	N	Three days of learning walks under natural conditions, as a control for the experiment;	Gaze direction

the full light spectrum without a polarization pattern had an influence on the ants' behavioral development, a UV-permeable plexiglass that diffused the skylight was installed. The skylight polarization pattern and the position of the sun was blocked using a UV-impermeable plexiglass with a sunshade (UVBS; **Figures 1B,C**). On the second day of marking a camera set-up was placed north to the nest entrance. Two cameras were installed: a 4K-camcorder (HC-X1000, Panasonic Corporation, Kadoma, Japan) that recorded learning walks of novices at 50 fps, and a Full-HD camcorder (HDR-CX330E, Sony Corporation, Minato, Japan) that recorded the nest area at 25 fps for the entire day. Every time an unmarked ant left the nest entrance, an observer positioned south of the experimental setup triggered recordings of the 4K-camcoder using the Panasonic Image App (Version 10.9.2, Panasonic Corporation, Kadoma, Japan) on a Sony Xperia Z1 smartphone (Sony Corporation, Minato, Japan). Since it was not possible to film through the diffusor (Dif), only observational data is available for this experimental trial.

Neuroanatomical Procedures

Anti-Synapsin Immunolabeling

On the third day of recording, novices that performed wide range learning walks reaching up the fence were captured under the filter setup and kept in the dark until the next day. This ensured, that the ants had performed several learning walks under the altered skylight conditions and that their brains had enough time to undergo structural changes (Stieb et al., 2012; Fleischmann et al., 2016, 2017; Schmitt et al., 2016). In addition, interior workers (DD) were collected from another nest in which, similar to the experimental nest, all ants leaving the nest were marked over three consecutive days. In order to get interior workers that had never seen daylight before, the nest was excavated in the night using red light. All ants were kept in a dark box until the next day.

To analyze neuroanatomical changes in the CX and MBs (all neuroanatomical nomenclature after Ito et al., 2014), the brains were stained using a primary antibody to synapsin (SYNORF1, kindly provided by E. Buchner, University of Würzburg, Germany) and a secondary antibody coupled to AlexaFluor 568 (A12380, Molecular Probes, Eugene, OR, USA) dye.

The ants were cooled down in a freezer and decapitated in the dark. Immediately afterwards the brains were carefully dissected and fixated in 4% formaldehyde in phosphate-buffered saline (PBS) for 1 day. The brains were then rinsed three times in PBS for 10 min, followed by one rinse in 2% Triton-X 100 solution in PBS and two rinses in 0.5% Triton-X solution, for 10 min each, to permeabilize cell membranes for antibody application on whole mount brains. To block unspecific binding sites, the brains were then incubated for 1 h at room temperature on a shaker in a 0.5% Triton-X 100 solution in PBS with 2% of Normal Goat Serum (NGS, Jackson ImmunoResearch Laboratories). Afterwards, the brains were incubated for 3 days in the refrigerator (∼4°) on a shaker with the primary anti-synapsin antibody from mouse. A solution with 2% antibody, 2% NGS and 0.5% Triton-X 100 in PBS was used. After incubation the brains were rinsed five times for 10 min each in PBS. Then the secondary antibody, an anti-mouse antibody from goat with an Alexa Fluor 568 dye (4% in PBS with 1% NGS), was incubated for 2 days in the refrigerator on a shaker. The brains were then rinsed again three times in PBS for 10 min each, before they were dehydrated using an ethanol serial dilution. For that, they were rinsed for 10 min in every step: 30%, 50%, 70%, 90%, 95% ethanol in water and two times in 100% ethanol. The dehydrated brains were then cleared in methyl salicylate (M-2047; Sigma-Aldrich, Steinheim, Germany).

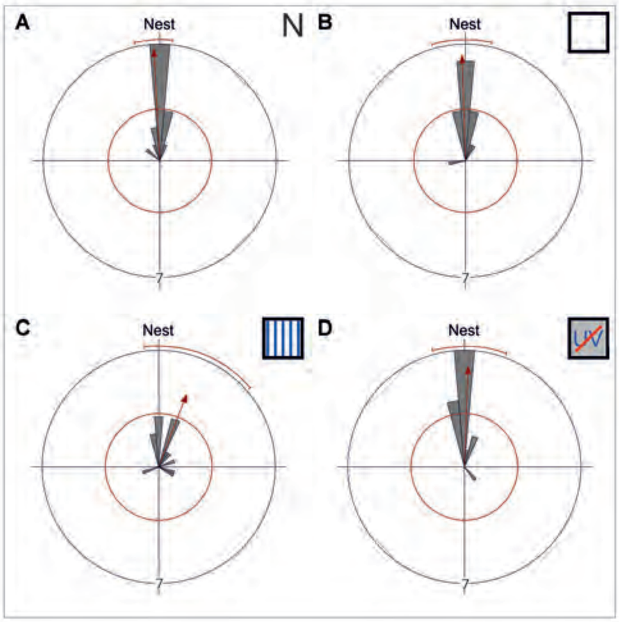

FIGURE 2 | Gaze directions during the longest stopping phases under different skylight conditions. Data are shown in gray and the corresponding statistics in red. The bins of the circular histogram include 10 degrees. The red circle indicates the critical value $\alpha = 0.05$ of the Rayleigh uniformity test. The red arrow indicates the r-vector pointing towards the mean direction. If the length of the vector exceeds the red circle the data is directed ($p < 0.05$). When the data is directed, a red line indicates the 95% confidence interval. If the expected direction (Nest ≙ 180°) lies within the confidence intervals limits, the data is directed towards the nest entrance. The outer circle indicates tic 7. Each data point is contributed by one back turn of one ant. **(A)** The mean gaze direction of the longest stopping phase in pirouettes during learning walks under natural/no filter conditions (N) is directed towards the nest entrance ($n = 15$). **(B)** The same is true for the mean gaze direction of the longest stopping phase under control conditions (UV100; $n = 15$) and **(C)** under an artificial, fixed polarization pattern (P; $n = 14$). **(D)** Even when excluded from all celestial information (UVBS; $n = 15$) the ants were able to gaze towards the nest entrance during the longest stopping phases. The mean angle and the angular variance did not differ between the four groups. For statistical details see text.

Anterograde Tracings of Neuronal Projections from the Medulla

To determine the neuronal projections via the asot in *C. noda*, projection neurons of the dorsal and ventral medulla (ME) were fluorescently stained in ants reared in laboratory colonies. The tracings of neuronal projections from the ME were performed

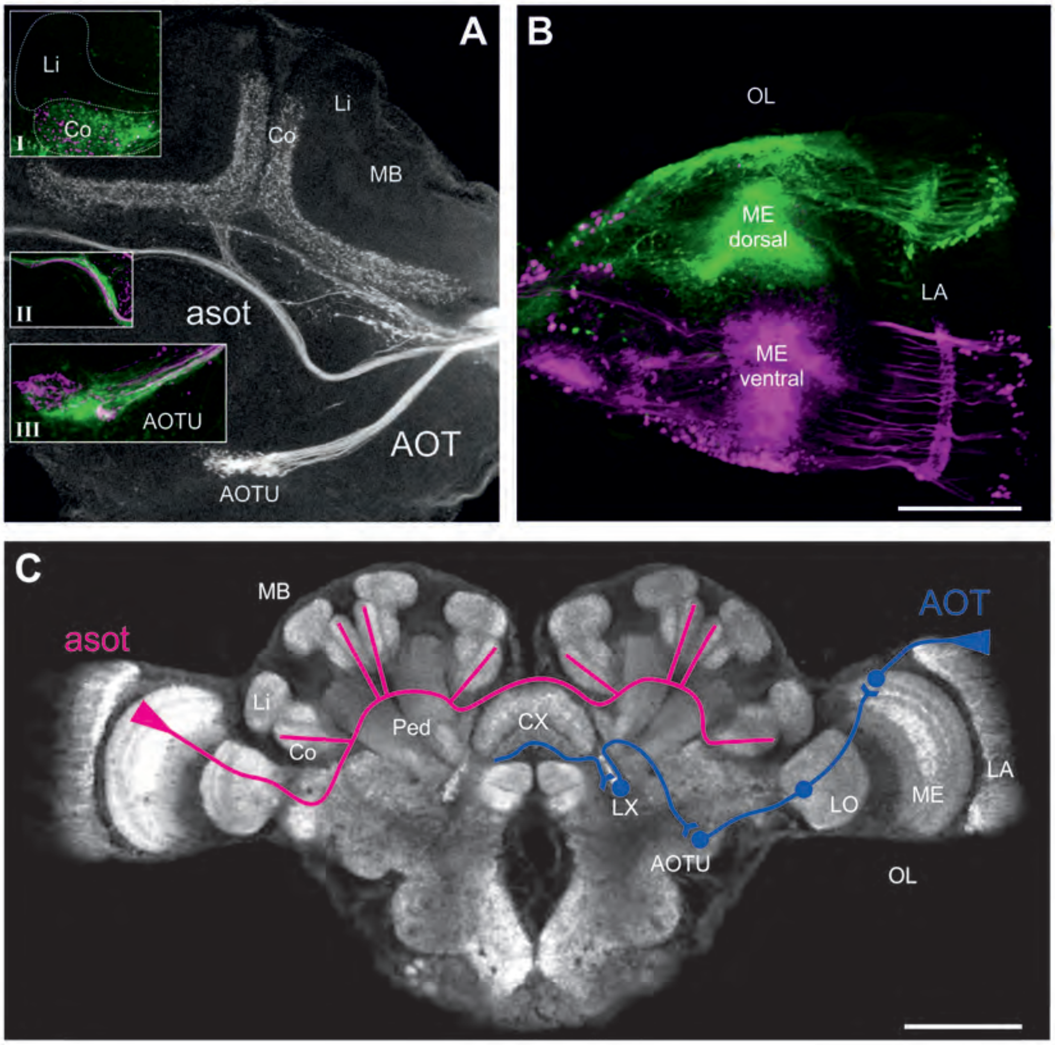

FIGURE 3 | Neuronal projections from the medualla (ME) via the anterior optical tract (AOT) and the anterior superior optic tract (asot) in the *Cataglyphis noda* brain. Anterograde tracings from focal dye injections the dorsal and ventral medulla (ME; microruby in magenta, Alexa 488 dextran in green, see under **B**): **(A)** Axon bundles from projection neurons in the medulla run anterior above the peduncle (Ped) and the central complex (CX) into the visual subregion of the mushroom body (MB) collar (Co) on both sides of the brain. Axonal projections from both the dorsal and the ventral ME run along the asot (inset **II**) into the Co. The most prominent input in the MB-calyx Co was found in injections into the dorsal ME (green) compared to those in the ventral ME (magenta) (inset **I**). Axonal projection from the ME also run into the anterior optical tubercle (AOTU) along the AOT. Z-projection from a stack of 27 images, 10x objective, 5 μm step size. Insets were taken with a 20x objective, 5 μm step size. **(B)** In the dorsal ME Dextran AlexaFloun488 (green) was injected using a glass capillary. In the ventral ME Dextran Tetramethylrhodamine (micro-Ruby) (magenta) was injected using a glass capillary. Images taken with a 10x objective, step size of 10 μm, stack of 19 images, zoom 2.65. The scale bar in **(B)**, also valid for **(A)**, is 100 μm. **(C)** Schematic depiction of the tracing of the asot (magenta) and the AOT (blue). The asot, as seen in the tracings in **(A)**, runs from the ME anterior above the peduncle and the CX into Co. The AOT (information combined with the one from Schmitt et al., 2016) runs from the dorsal rim of the lamina (LA) to the dorsal rim of the ME, and from there via the LO to the AOTU to be relayed further to the lateral complex (LX). The anterior CX pathway terminates in the lower half of the ellipsoid body (EB) of the CX (Schmitt et al., 2016). The confocal scan of the *C. noda* brain shows an anti-synapsin labeled brain, similar to the staining procedure used for the neuroanatomical analyses. The scale bar is 200 μm.

using similar methods as described in detail in Yilmaz et al. (2016). Ants were cooled and fixed with clay. A small window was cut in the head capsule, and the brain was rinsed with cooled ant ringer solution. Using a thin glass capillary, dextran tetramethylrhodamine (micro-Ruby, D-7162, Molecular Probes, Eugene, OR, USA) and Dextran AlexaFluor488 (D-22910, Molecular Probes, Eugene, OR, USA) were focally inserted in the dorsal and ventral medulla. The brain was then rinsed with ringer solution and the head capsule was covered with a thin piece of Parafilm to prevent the brain from drying out. The dyes were allowed to be transported by incubating the ants for 3 h at room temperature in a dark box with high humidity. Afterwards, the brains were dissected in cooled ringer solution and fixated in 4% formaldehyde in PBS overnight. The brains were rinsed five times in PBS for 10 min each before they were dehydrated using an ethanol serial dilution. For that, they were rinsed for 10 min, each step: 30%, 50%, 70%, 90%, 95% in water, and two times 100% ethanol. The dehydrated brains were then cleared and mounted in methyl salicylate. Finally, the brains were digitized in the confocal laser scanning microscope (see below) using a 20×- or 10×-objective and step sizes of 5 μm or 10 μm.

Data Analyses

High-Speed Video Analyses

The 4K-videos obtained from the experiments were converted into image stacks using the Free Video to JPG Converter (v. 5.0.99 build 823, DVDVideoSoft, DIGITAL WAVE LTD., London, UK). Subsequently, the pirouettes (tight back turns Fleischmann et al., 2017) performed by novices were analyzed frame by frame using the MATLAB (2015a, The MathWorks Inc., Natick, MA, USA) application DIGILITE (Jan Hemmi and Robert Parker, The Australian National University, Canberra, Australia). For this, the positions of the mandibles and the thorax were marked manually in each frame. Additionally, the position of the nest entrance and the north direction were marked. With these coordinates the gaze direction relative to the nest entrance of the ants during their back-turns was determined. The direction of the nest entrance was defined as 180°. Stopping phases during the pirouettes were defined as in Fleischmann et al. (2017), and the longest of these stopping phases was used to test the directedness of the back turns.

Neuroanatomical Analyses

For microscopic analyses, the brains that had been dissected and histochemically treated in our field laboratory were transferred to the University of Würzburg using a refrigerator unit (∼4°C). A confocal laser scanning microscope (Leica TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany) was used for scanning the brains as image stacks at a step size of 5 μm. We used the 10×-objective for overviews with 2.5 optical zoom NA imm (for CX), the 20×-objective with 2.7 optical zoom NA imm for the MB calyx, and the 63×-objective with 2.0 optical zoom NA imm for detailed scans in the lip (Li) and collar (Co) of the MB calyx. Subsequently, the volumes of the different components of the CX (fan-shaped body (FB), ellipsoid body (EB), protocerebral bridge (PB), noduli (No)) and of the MB calyx (Li, Co) were analyzed using the 3D-reconstruction software Amira (Amira 6.0.0, FEI Company, Hilsboro, OR, USA). In addition, synaptic complexes (microglomeruli, MG) were quantified in the visual and olfactory subregions of the MB calyx (Li, Co) using a modified version of the protocol by Groh et al. (2012; for further details, see Rössler et al., 2017). The CX, MB and other major neuropils were easily distinguishable in anti-synapsin labeled whole mount brains (**Figure 3C**), and based on tracings (Yilmaz et al., 2016, **Figures 3A,B** for *C. noda*). MB-calyx MG were quantified by counting the anti-synapsin labeled synaptic boutons in a defined volume of 1000 μm^3. The MG density was then calculated by averaging multiple volumes of interest in the two subregions (three in the Co, four in the Li) as numbers of MG per μm^3 following the protocol by Groh et al. (2012) and Muenz et al. (2015). From these numbers the total number of MG per calyx subdivisions was estimated by multiplying the MG densities by the volume of the corresponding neuropil. The ants used in this experiment had a median thorax length of 4.24 mm, ranging from 3.18 mm to 5.58 mm. Thorax length correlates with body size (Vowles, 1954) and, therefore, also with total brain size (Wehner et al., 2007). Since we did not find a correlation between thorax length and the analyzed neuropils of interest (Spearman roh test (α = 0.05): CX: n_{CX} = 45, p_{CX} = 0.545, r_{CX} = 0.093; MB: n_{MB} = 43, p_{MB} = 0.058, r_{MB} = 0.291), absolute volumes and MG numbers were used in this study. These results are coherent with results obtained using head width as a measure for body size in *C. fortis* (Stieb et al., 2010). As no major group-specific differences in thorax lengths were apparent (Supplementary Figure S1), comparisons were made without corrections for group bias in overall brain size.

Statistical Analyses

The gaze directions were grouped into 10°-bins as previously done by Fleischmann et al. (2017). The circular statistical software Oriana (Kovach Computing Services, Anglesey, UK) was used to check with the Rayleigh test whether the data was randomly distributed. If the gaze directions were directed (α = 0.05), we calculated the 95% confidence interval to check whether the expected direction (nest entrance: 180°) was within the limits. The mean angle and the angular variance were compared between the groups using a Mardia-Watson-Wheeler multisample test (α = 0.05).

In the neuroanatomical studies, the volume between the different groups (DD, UVBS, Dif, P, UV100) within each neuropil (CX, Co, Li) was compared using the Kruskal-Wallis-test (α = 0.05). In cases when a difference between the groups occurred, a *post hoc* pairwise comparison between DD and the other groups was performed using a Mann-Whitney U-test with Bonferroni correction. A critical value of α = 0.05 was used (after Bonferroni correction: α = 0.0125).

RESULTS

Gaze Direction Analyses and Behavioral Observations Under Different Skylight Conditions

While initially leaving their nest under natural conditions (N), *C. noda* walked in small loops around their nest entrance, similar as shown earlier (Fleischmann et al., 2017). These learning walks were repeatedly interrupted by characteristic turns, so called voltes and pirouettes. During the latter, the ants performed multiple stopping phases (n = 15, 4 ± 1.75, median ± IQR) with the longest stopping phases directed towards the nest entrance (Rayleigh Uniformity Test: Z_0 = 13.856, n = 15, $p < 0.001$; 95% Confidence Interval (−/+) 167.9°/186.0°; Mean: 177.0°; **Figure 2A**). The gaze direction during the longest stopping phases was directed towards the nest entrance when the experimental setup was installed using a UV-light

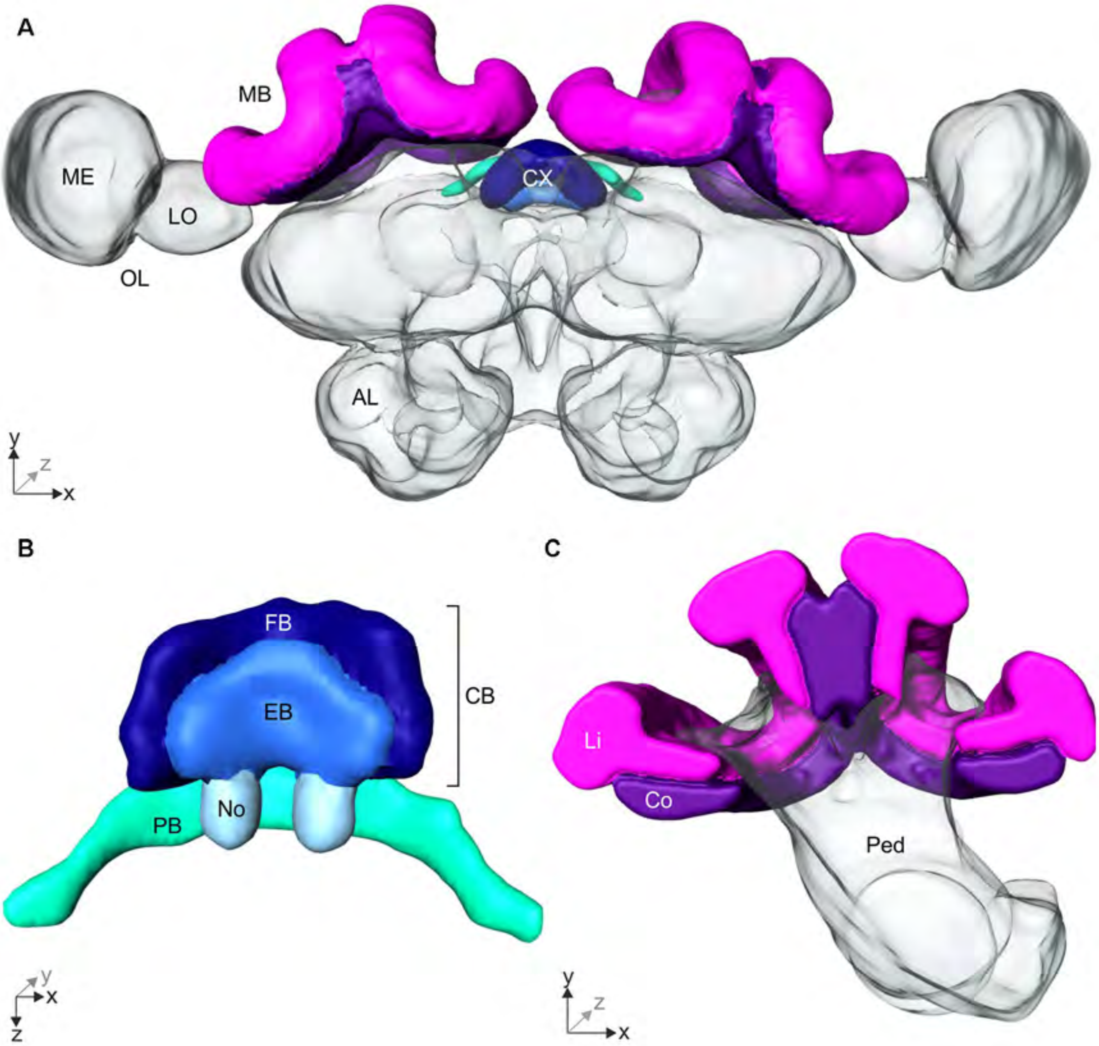

FIGURE 4 | 3D-reconstruction of the *Cataglyphis noda* brain. With the 3D-reconstruction software Amira the neuropils of the *C. noda* brain were manually reconstructed from the image stack obtained by the confocal laser scanning microscope. **(A)** 3D-reconstruction of a whole *C. noda* brain. To analyze the influence of celestial information during learning walks on neuroplasticity, the terminal stages of two visual pathways were reconstructed. The AOT transfers visual information, including polarization information, into the central complex (CX, shades of blue). The CX is located at the midline of the ant brain. Via the asot visual information is transferred to the mushroom body calyces (MB, magenta). Additionally, antennal lobes (AL), and optical lobes (OL) with the medulla (ME) and lobula (LO) are labeled. **(B)** The CX comprises several neuropils: The central body (CB, dark and light blue) is located most anterior. It consists of the large fan-shaped body (FB, dark blue) and the smaller ellipsoid body (EB, light blue), which is covered by the FB dorsally. Behind the CB, two globular neuropils, the noduli (No, pale blue) are located. Dorsally to that and slightly detached from the CB, the protocerebral bridge (PB, green) spans in a bridge-like shape between the mushroom bodies (MB). **(C)** The MB calyx includes the visual input region, the collar (Co, violet) and the olfactory input region, the lip (Li, magenta). They are located at the dorsal rim of the peduncle (Ped). Scale bars, **(A)** 200 μm; **(B,C)** 100 μm.

permeable filter as a control (UV100; Rayleigh Uniformity Test: $Z_0 = 12.306$, $n = 15$, $p < 0.001$; 95% Confidence Interval (−/+) 163.9°/192.55°; Mean: 178.2°; **Figure 2B**). When the natural skylight polarization pattern was altered to a linear one that did not change over the day (P) the overall structure of the walks remained unchanged and the gazes during the longest stopping phases were clearly directed towards the nest entrance (Rayleigh Uniformity Test: $Z_0 = 6.189$, $n = 14$, $p = 0.001$; 95% Confidence Interval (−/+) 173.1°/229.1; Mean: 201.1°; **Figure 2C**). One analyzed pirouette under P did not contain a stopping phase and therefore was not included in the circular statistics. After the learning walks had taken place for several days under this fixed polarization pattern, the polarization filter was rotated by either by 90° or in two steps of 45°. From visual observations we noticed that the sudden changes in the polarization pattern above the nest entrance seemed to increase the number of naïve ants performing learning walks shortly after the change took place (experimental day with stationary linear polarization pattern number of learning walks: $n = 71$ vs. experimental day with stepwise rotated (45°

every hour) linear polarization pattern number of learning walks: $n = 277$). When learning walks were performed under a diffused polarization pattern (Dif) no apparent changes in learning walk patterns compared to natural conditions could be observed. For the Dif conditions, further quantitative video analyses were not possible since we could not record through the diffusor. Nevertheless, more than 100 pirouettes, all directed towards the nest entrance, were observed during the three experimental days. However, even learning walks that were performed under the exclusion of any sky compass information by blocking UV-light, which is necessary for the ants to perceive the polarization pattern (Duelli and Wehner, 1973), and, at the same time, by excluding the position of the sun by using a sunshade (UVBS) were not altered in their overall structure compared to learning walks under natural conditions. The longest stopping phase of pirouettes under UVBS conditions was directed towards the nest entrance (Rayleigh Uniformity Test: $Z_0 = 11.406$, $n = 15$, $p < 0.001$; 95% Confidence Interval (−/+) 166.4°/200.0°; Mean: 183.2°; **Figure 2D**). The mean angle or the angular variance did not differ between all experimental groups (Mardia-Watson-Wheeler multi sample test: $W = 6.124$; $n_{\mathrm{N}} = 15$; $n_{\mathrm{UV100}} = 15$; $n_{\mathrm{P}} = 14$; $n_{\mathrm{UVBS}} = 15$; $p = 0.375$).

The AOT and asot in the *Cataglyphis* Brain

To investigate visual pathways to high-order integration centers in *C. noda* brains, we performed focal dye injections and anterograde neuronal tracings of neuronal projections from the dorsal and ventral medulla (ME; **Figure 3B**). This clearly revealed neuronal projections via the asot and via the AOT (**Figure 3A**). From 16 dye injected brains, three were successfully double stained (dorsal and ventral ME), three showed tracings from the dorsal ME only, and two from the ventral ME only. In all tracings, the asot projected from the ME anteriorly above the peduncle and the central complex (CX), bilaterally into the collar (Co) of the medial and lateral branches of the MBs (**Figures 3A,C**). Visual inspection of all tracings indicated that axonal projections via the asot from the dorsal ME were more prominent compared to the sparser projections and terminal branches from the ventral ME in the MB Co ($n = 8$; **Figure 3A**, inset I).

All tracings from the dorsal and ventral ME revealed projections to the anterior optic tubercle (AOTU) via the AOT (**Figures 3A,C**). The AOT was previously described in detail for *C. fortis* (Schmitt et al., 2016) by tracing projections only from the dorsal rim area of the lamina LA and ME. From there further stages are the lobula (LO), the AOTU, the lateral complex (LX) and finally the lower half of the EB of the CX (**Figure 3C**; for locust: Homberg et al., 2011; for *C. fortis*: Schmitt et al., 2016). Interestingly, our differential tracings from the dorsal and ventral ME revealed a clear pattern in the AOTU with a clear separation of ventral and dorsal projections in the upper unit of the AOTU and a mixed pattern in the lower part of the AOTU (**Figure 3A**, inset III).

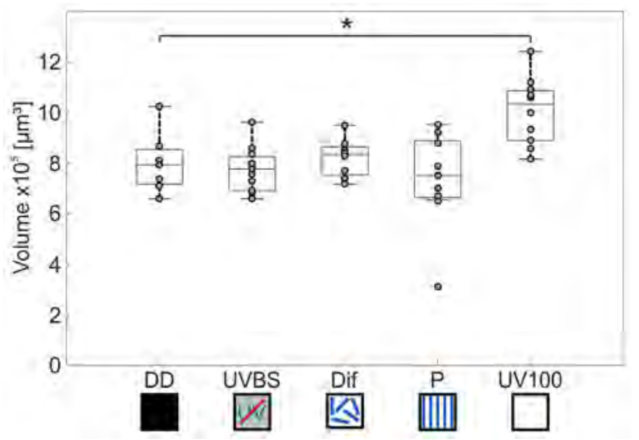

FIGURE 5 | Volume changes of the CX after 3 days of learning walks dependent on celestial information. The central line of each boxplot depicts the median of the data. The upper and lower limits of the boxes show the 25th and 75th percentiles, while the whiskers extend to the extreme data points without outliers. All data points (including outliers) are plotted as gray circles. A difference between the groups can be found using a Kruskal–Wallis test. With the Mann-Whitney U-test with a Bonferroni correction the data was *post hoc* compared to the DD group. The asterisk indicates that data is significantly different (after correction $p < 0.0125$) from the DD group. The central complex shows a volume increase compared to interior workers (DD; $n = 7$) only if the ants perceived the UV mediated natural polarization pattern that changes over the day (UV100; $n = 10$). If the polarization pattern was altered, either by diffusion (Dif; $n = 8$) or by a linear polarization filter (P; $n = 9$), no change in the volume of the CX occurred compared to DD. Similarly, when ants were excluded from any celestial information (UVBS; $n = 11$), no volume increase occurred. For statistical details and further explanations, see text.

Influence of Manipulated Skylight Input during Learning Walks on Neuronal Plasticity in the CX and MB

We investigated the influence of skylight manipulations during learning walks on neuronal changes in the terminal stages of the AOT and asot. The brains of ants that had participated in the behavior tests and had performed several days of learning walks under normal or altered skylight conditions were analyzed using 3D-reconstructions of the CX and MB (**Figure 4**), and quantifications of synaptic complexes in the MB. For comparison, brains of ants that had not yet performed learning walks (DD) were analyzed.

Volumetric Changes in the CX

The AOT transfers visual information into the CX (**Figure 3C**). The CX comprises several neuropils (**Figure 4B**): The central body (CB) is located most anterior and consists of the large FB and the smaller EB, which is covered by the FB dorsally. Behind the CB, two globular neuropils, the No, are located. Dorsally to that and slightly detached from the CB, the PB spans in a bridge-like shape between the MBs (**Figure 4B**). Comparing the CX of the ants that had previously participated in the behavioral studies (DD, UVBS, Dif, P, UV100), showed a statistically significant difference between their CX volumes (Kruskal–Wallis test: CX Volume: $\chi^2_4 = 16.38$; $n = 45$; $p = 0.0046$; **Figure 5**). Compared to the CX of interior workers (DD) the CX

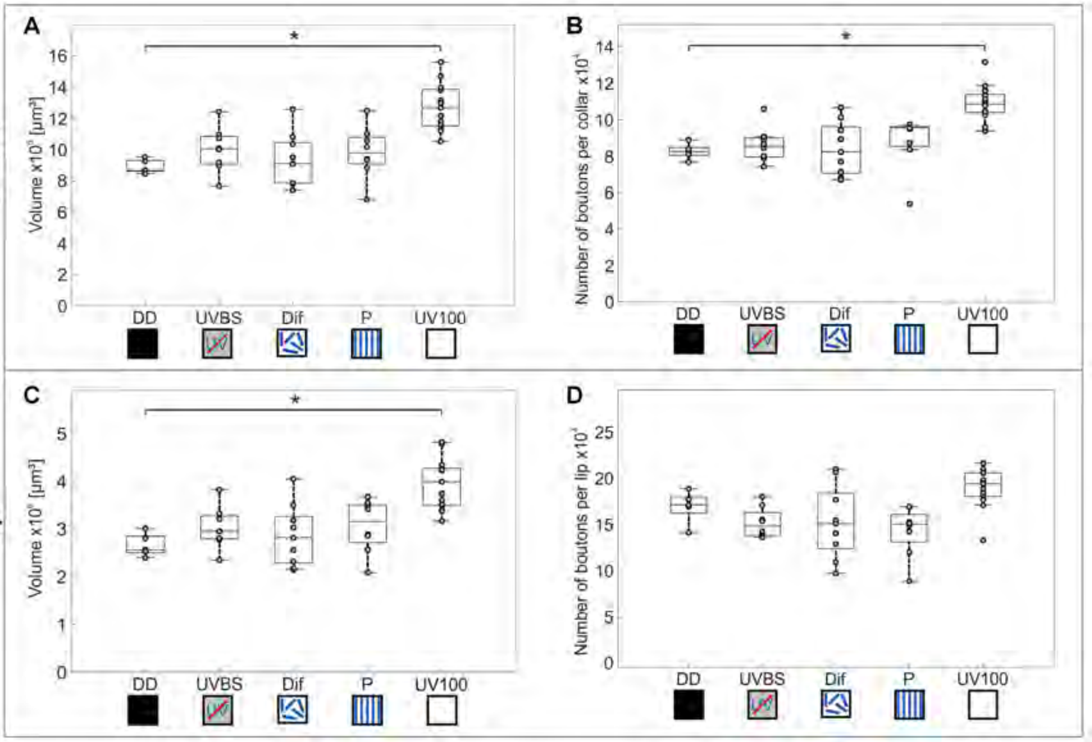

FIGURE 6 | Volume changes and changes in numbers of synaptic complexes in MB calyx subdivisions. The central line of each boxplot depicts the median of the data. The upper and lower limits of the boxes show the 25th and 75th percentiles, while the whiskers extend to the extreme data points without outliners. All data points (including outliers) are plotted as gray circles. To find a difference between the groups, a Kruskal–Wallis test was used (α = 0.05). With the Mann-Whitney U-test with a Bonferroni correction the data was *post hoc* compared to the DD group if the Kruskal–Wallis test indicated a difference. The asterisk indicates data significantly different (after correction $p < 0.0125$) from the DD group. **(A)** In the MB-calyx Co, a significant volume increase compared to DD ($n = 5$) occurred only when the learning walks were conducted under the natural UV mediated changing polarization pattern (UV100; $n = 13$). **(B)** Similarly, the total number of synaptic boutons per calyx in the Co only increased when the learning walks were performed under UV100 compared to DD. **(C)** In the MB-calyx Li, a volume increase occurred only under UV100 conditions compared to DD, similar to the conditions in the Co. **(D)** However, in the Li no change in the total number of synaptic boutons per calyx occurred under any conditions. No significant differences in the MB occurred between DD and UVBS ($n = 8$), Dif ($n = 9$), or P ($n = 8$). For statistical details and further explanations, see text.

in brains of *C. noda* that had performed several learning walks under a naturally changing polarization pattern (UV100) showed a volumetric increase (Mann-Whitney U-test with Bonferroni correction: DD vs. UV100, $Z_4 = -2.6837$; $n_{\mathrm{DD}} = 7$; $n_{\mathrm{UV100}} = 10$; $p = 0.0073$). The volumetric increase in the CX was absent compared to DD when the ants performed their learning walks under restricted skylight conditions including an artificially fixed linear polarization pattern (P) (Mann-Whitney U-test with Bonferroni correction: DD vs. P, $Z_3 = 0.4234$; $n_{\mathrm{DD}} = 7$; $n_{\mathrm{P}} = 9$; $p = 0.6720$), a diffused polarization pattern (Dif) (Mann-Whitney U-test with Bonferroni correction: DD vs. Dif, $Z_2 = -0.7522$; $n_{\mathrm{DD}} = 7$; $n_{\mathrm{Dif}} = 8$; $p = 0.4519$), and without polarization pattern and information about the position of the sun (UVBS; Mann-Whitney U-test with Bonferroni correction: DD vs. UVBS, $Z_1 = 0.5434$; $n_{\mathrm{DD}} = 7$; $n_{\mathrm{UVBS}} = 11$; $p = 0.5869$). The same statistical relationships were found for the volume of the CB only, which includes the ellipsoid (EB) and the fan-shaped body (FB). When comparing the subunits (EB, FB, PB and No) individually, the same tendency was found, but was not statistically significant.

Volumetric Changes in the MB and Plasticity of Synaptic Complexes

Comparison of the volume and the numbers of synapsin labeled synaptic boutons in the MB calyx Co (**Figure 4C**) revealed a significant difference between the experimental groups of the behavior essay (Kruskal–Wallis test: Co Volume: $\chi^2_4 = 22.43$; $n = 43$; $p = 0.00016$; Co No. Synapses: $\chi^2_4 = 23.06$; $n = 43$; $p = 0.00012$; **Figures 6A,B**). Only ants that had performed several learning walks under a naturally changing skylight polarization pattern (UV100) showed an increase in the volume of the MB calyx Co and the estimated total number of synapses per calyx compared to ants that had not yet performed learning walks (DD; Mann-Whitney U-test with Bonferroni correction: Co Volume: DD vs. UV100, $Z_4 = -3.1543$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UV100}} = 13$; $p = 0.0016$; Co No. Synapses: DD vs. UV100, $Z_4 = -3.1543$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UV100}} = 13$; $p = 0.0016$). All groups that had performed learning walks under restricted skylight conditions did not show a significant increase compared to DD, neither in the volume nor in the total number of MG synaptic complexes per calyx in the MB calyx Co (Mann-Whitney

U-test with Bonferroni correction: CO Volume: DD vs. UVBS, $Z_1 = -1.5370$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UVBS}} = 8$; $p = 0.1243$; DD vs. Dif, $Z_2 = -0.2667$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{Dif}} = 9$; $p = 0.7897$; DD vs. P, $Z_3 = -1.5370$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{P}} = 8$; $p = 0.1243$; Co No. Synapses: DD vs. UVBS, $Z_1 = -0.6587$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UVBS}} = 8$; $p = 0.5101$; DD vs. Dif, $Z_2 = 0$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{Dif}} = 9$; $p = 1$; DD vs. P, $Z_3 = -1.8298$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{P}} = 8$; $p = 0.0673$). The volume of the Li also differed significantly between groups (Kruskal–Wallis test: Li volume: $\chi^2_4 = 20.08$; $n = 43$; $p = 0.00048$; **Figure 6C**). The volume was increased significantly compared to DD in ants that had performed several learning walks under UV100 conditions (Mann-Whitney U-test with Bonferroni correction: Li Volume: DD vs. UV100, $Z_4 = -3.1543$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UV100}} = 13$; $p = 0.0016$). No difference in the Li volume occurred between DD and the other groups, (Mann-Whitney U-test with Bonferroni correction: Co Volume: DD vs. UVBS, $Z_1 = -1.2443$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UVBS}} = 8$; $p = 0.2134$; DD vs. Dif, $Z_2 = -0.5333$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{Dif}} = 9$; $p = 0.7897$; DD vs. P, $Z_3 = -1.5370$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{P}} = 8$; $p = 0.1243$). In contrast to the visual MB subregion (Co), however, there was no significant difference compared to DD based on pair-wise comparison of the total number of MG synaptic complexes per calyx in the MB olfactory Li, despite the groups not coming from the same distribution (Kruskal–Wallis test: Li No. synaptic complexes: $\chi^2_4 = 15.81$; $n = 43$; $p = 0.0033$; Mann-Whitney U-test with Bonferroni correction: Li No. synaptic complexes: DD vs. UVBS, $Z_1 = 1.5370$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UVBS}} = 8$; $p = 0.1243$; DD vs. Dif, $Z_2 = 0.9333$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{Dif}} = 9$; $p = 0.3506$; DD vs. P, $Z_3 = 1.9762$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{P}} = 8$; $p = 0.0481$; DD vs. UV100, $Z_4 = -1.9715$; $n_{\mathrm{DD}} = 5$; $n_{\mathrm{UV100}} = 13$; $p = 0.0487$; **Figure 6D**).

DISCUSSION

Celestial Information Is Not Necessary for the Look Back to the Nest Behavior

In the beginning of their foraging careers, *C. noda* perform learning walks that are repeatedly interrupted by turns with several stopping phases. The longest stopping phases are accurately directed towards the nest entrance (Fleischmann et al., 2017). It has previously been suggested that ants may use path integration information to align their back turns (Graham et al., 2010; Müller and Wehner, 2010). We used this conspicuous feature in the learning walks of *C. noda* as an easily quantifiable behavior readout in skylight manipulation experiments to ask whether celestial cues may serve as a reference system to align gaze direction. Our results demonstrate that neither an artificial (P) nor a diffused (Dif) polarization pattern disturbed the directedness of the longest stopping phases toward the nest entrance. Even with complete exclusion of the polarization pattern and the position of the sun (UVBS), the ants were still able to perform the look back to the nest entrance behavior. This strongly suggests that the celestial compass—providing the directional component of the path integration system during foraging (review: Wehner, 2003)—is not the system of reference used by ants to initially align the gaze direction during naïve learning walks. Our results underline the robustness and importance of the mechanism that is used to align the gaze direction during the longest stopping phases.

Possible Reference Systems for the Look Back to the Nest Behavior

As our results show that the celestial compass does not provide a reference system used during learning walks, other possibilities for the compass component of the path integrator have to be considered. A potential candidate could be the visual landmark panorama. Schultheiss et al. (2016) recently demonstrated that UV-light plays a crucial role for the use of the landmark panorama. However, our results show that *C. noda* was still able to look back to the nest entrance during learning walks under blocked UV-light spectrum (UVBS). Furthermore, the panorama information is not yet known or memorized in ants during naïve (first) learning walks and requires the completion of several learning walks (Fleischmann et al., 2016). The ants might also use nest odors to detect the direction of the nest. *C. fortis* were shown to use olfactory landmark cues near the nest (Steck et al., 2009). However, as the ants conduct their learning walks in increasing distances and in all compass (including upwind) directions away from the nest (Fleischmann et al., 2016), olfactory cues are not reliable during the entire learning walk sequences. The ants also walk cross wind in order to approach odor sources, in particular prey items during foraging (Wolf and Wehner, 2000; Buehlmann et al., 2014). This behavior has not become evident in learning walks, and as the ants perform pirouette-like turns all-around the nest entrance, cross wind orientation seems highly unlikely. Finally, the ants could use intrinsic (idiothetic) orientation mechanisms. Such mechanisms however, would be highly prone to cumulative errors (Müller and Wehner, 1994). An error in the gaze direction during the longest stopping phase of pirouettes would lead to a snapshot taken into the wrong direction. This could easily lead to serious errors in foragers, but also during learning walks with extended lengths. A more promising candidate for an initial reference system for the compass component of the path integrator during learning walks of *C. noda* is the geomagnetic field. This had already been suggested for the learning flights of bumblebees (Collett et al., 2013). Furthermore, *C. noda* was shown to learn magnetic landmarks (Buehlmann et al., 2012). Although the magnetic field strength, in these experiments, was far above the natural geomagnetic field, it appears likely that the ants possess a magnetic sense that could be used for the initial calibration of navigational information. A potential role of a magnetic sense has also been suggested for other ants (fire ants: Anderson and Vander Meer, 1993; leaf-cutter ants: Banks and Srygley, 2003; wood ants: Çamlitepe and Stradling, 1995; for a review see: Wajnberg et al., 2010). However, so far no use of the geomagnetic field for navigation, in particular as compass information for path integration, has been described in ants, neither for experienced foragers during their foraging runs, nor for learning walks in novices. Therefore, at this point the question regarding an initial reference system for the alignment of gaze directions to acquire and calibrate navigational information during learning walks has to remain open.

Visual Pathways in the *C. noda* Brain

To be used as navigational information, the visual information perceived by the ants during learning walks needs to be relayed to and processed in higher integration centers of the brain. Using anterograde tracing techniques, two prominent visual pathways become apparent in *C. noda*. Visual information from the ME is transferred bilaterally to the MB collars of the medial and lateral MB calyces, very similar to the projections found in other Hymenoptera (Gronenberg, 2001; Yilmaz et al., 2016). In *Drosophila* only a very small subset of visual neurons transfers information from the OL to the MB calyx (Vogt et al., 2016). This may suggest that this pathway is highly conserved across insects, but the number of neurons and their projection patterns are adapted to the visual ecology of individual species (Groh et al., 2014; Vogt et al., 2016; Yilmaz et al., 2016). One interesting feature in *C. noda* is that axonal projections from the dorsal ME appear more extensive compared to projections from dye injections into the ventral ME. This may indicate that the dorsal retina and celestial view aspects are more prominently represented in the MB calyx Co compared to terrestrial aspects from the lower part of the compound eye. More focal injections, also along the horizontal axis, are needed to further analyze this. In *C. fortis* the AOT was shown to house projections from the dorsal most regions of the medulla indicating that polarization information from the dorsal rim area of the eye is transferred via this pathway to the AOTU and the LX into the lower half of the EB of the CX (Schmitt et al., 2016), similar to the conditions found in locusts (Homberg et al., 2011). Our results show that also the ventral region of the medulla is relayed to the upper and lower part of the AOTU. Next we tested whether the high-order sensory integration centers (MB, CX) express neuroplasticity related to the quality of celestial information experienced during learning walks.

Natural Polarization Pattern Is Necessary for a Volume Increase in the CX

Although our manipulations of celestial information did not significantly alter the learning walk behavior, the restriction of skylight information interfered with neuroanatomical changes in the CX. A volume increase in the CX as compared to DD occurred only when the learning walks had been conducted under the full spectrum including UV-light and the naturally changing polarization pattern. Exposure to the full light spectrum including UV-light with an artificial, fixed polarization pattern (P) or without a usable polarization pattern (Dif) did not lead to a CX volume increase. In contrast, a volume increase in the CB of *Drosophila* occurs after the flies were exposed to UV-light (Barth and Heisenberg, 1997). However, in that case *Drosophila* did not perceive a natural light and polarization pattern. In *C. noda* the exclusion of UV-light, and thereby the reception of the polarization pattern during learning walks, prevented volumetric chances of the CX. It is not possible with the methods available to count synapses within subunits of the CX. Therefore, we only analyzed volumetric changes in the CX. Previous studies on large synaptic complexes (giant synapses, GS) in the lateral complex (LX) along the sky-compass pathway of *C. fortis* revealed a significant increase of GS numbers depending on exposure to the UV part of the light spectrum (Schmitt et al., 2016). Therefore, it seems likely that the volume increase in the CX is also due to an increase in the number of synapses along this pathway. This increase was found to be significant in the CB units, i.e., the input region of the CX. Within the CX, in particular the PB, the skylight polarization direction is represented in a map-like manner (Pfeiffer and Homberg, 2014), and it has been shown through computational investigation that the CX is able to store spatial information (Fiore et al., 2017). Whether the neuroanatomical changes we found in the CX are triggered by appropriate sensory exposure or following the formation of spatial memory is an interesting question that needs to be investigated in a more focused approach. The CX is also involved in higher order control of movement of the limbs (Strauss, 2002; Martin et al., 2015), landmark orientation, and angular path integration (Seelig and Jayaraman, 2015). All this makes the CX a well suited neuropil to link polarization information to other stimuli mediating directional information important for navigation, for example other terrestrial reference systems.

Sensory Experience of a Natural Polarization Pattern Is Necessary for an Increase in the Number of Synaptic Complexes in the Visual Subregions of the MB Calyx

Similar to the results just described for the CX it was only under exposure to the naturally changing UV polarization pattern that a volume increase was found in the MB-calyx of ants that had performed their learning walks. Kühn-Bühlmann and Wehner (2006) had previously shown an increase in the MB volume of experienced (aged) foragers compared to dark reared ants of age-controlled *Cataglyphis bicolor*. In our study, we focused on the transition phase between interior worker (DD) and forager. Our data suggests that a volume increase in the MBs occurs already during learning walks and that it is dependent on the presence of the natural polarization pattern (UV100). A net increase of MB synaptic complexes was found only in the visual input region. As the MB is a higher order integration center involved in learning and memory, this may indicate that the increase in MG numbers is related to visual experience. Computer simulations by Ardin et al. (2015) suggest that the large synaptic capacity of visual subregions in ant MBs are well suited for the storage of visual snapshots underling the potential role of the MBs for learning and memorizing panoramic landmark cues during learning walks. Studies by Stieb et al. (2010, 2012) have shown that the MB Co expresses light-induced and age-dependent changes in MG numbers in *C. fortis*. Stieb et al. (2010) also showed a volume increase in the Co after exposure to full spectrum light accompanied by a decrease in MG densities. Furthermore, studies in the honeybee (Hourcade et al., 2010) and leafcutter ants (Falibene et al., 2015) showed that the formation of stable long-term olfactory memory leads to an increase in the density and number of MG in the Li. In contrast to the laboratory and partly restrained conditions in these experiments, the ants used in our study were allowed to perform their natural behaviors in their natural habitat under natural or altered skylight conditions. Therefore, a mix of both effects—the first exposure to light and long-term memory formation following learning, might be expected in our experimental ants. As UV-light is crucial for learning terrestrial landmarks (Schultheiss et al., 2016), an increase in synaptic complexes could be expected in the presence of UV-light, even without a naturally changing polarization pattern (Dif). Our data shows that a volume increase in the Co was absent in ants that had performed their learning walks under the full light spectrum, but without a usable polarization pattern (Dif) or with an artificial, fixed polarization pattern (P). Only when ants perceived a full spectrum including UV light together with a naturally changing polarization pattern, an increase in the volume and number of MG occurred in the MB calyx Co. No such effect was seen in MB collar MG of honeybees after a fine color discrimination task (Sommerlandt et al., 2016) indicating that only certain parameter combinations may lead to measurable effects of structural synaptic plasticity. The increase in the estimated MG numbers in the MB-calyx Co indicates an outgrowth of new presynapses during learning walks under natural skylight—a process similar to what has been observed after the formation of long-term memory (Hourcade et al., 2010; Falibene et al., 2015).

Due to the prominent role of path integration, *Cataglyphis* have to calibrate their internal skylight compass to the solar ephemeris (the season- and place-specific course of the sun over the day) at the beginning of their foraging career (Wehner and Müller, 1993). A panoramic- and celestial snapshot based mechanisms based on long-term memory in the visual MBs might play a role in this initial calibration. Similarly, short term learning of celestial snapshots was recently suggested for sky-compass orientation in dung beetles (el Jundi et al., 2016). When the skylight polarization pattern, however, does not change over the day (P), is diffused (Dif), or is not available (UVBS), it would not make sense to take and store celestial snapshots. To store new celestial information and thereby fine tune an internal template of the solar ephemeris function makes only sense if the polarization pattern changes compared to a fixed reference system. This hypothesis is also backed up by our observation that the number of learning walks drastically increased when the linear polarization filter was rotated. Analyzing neuroanatomical changes in ants that have performed learning walks under such a systematically changed artificial polarization pattern would allow for a deeper insight into the correlation shown so far.

The present study represents a first step of probing potential effects of learning walks on neuroplasticity. We started this combined field and laboratory study by focusing on the terminal projection areas of two prominent visual pathways in the CX and MB. To obtain a more comprehensive understanding of the total extent of learning-walk induced neuroplasticity, future investigations will have to include more extensive neuroanatomical analyses of all major brain neuropils, for example analyzing their volume relationships, synapse densities (whenever feasible), also in relation to overall brain volumes—for example like it was done in recent volumetric analyses of brains in migratory and solitary locusts, or migratory and non-migratory moths (Ott and Rogers, 2010; de Vries et al., 2017). In the same line, as previous work in *Camponotus* ants (Yilmaz et al., 2016) and in *Drosophila* (Barth and Heisenberg, 1997) show that the optic lobes undergo plastic changes after artificial light exposure, future studies on learning-walk induced neuroplasticity in *Cataglyphis* ants should include neuropils peripheral to the CX and MB, like the optic lobes, the AOTU and the lateral complex.

CONCLUSION

Neither the polarization pattern, or other information from UV-light input, nor the position of the sun are necessary for *C. noda* to align their gaze directions during the longest stopping phase of pirouettes in learning walks. Thus, the celestial compass as part of the path integrator does not provide the ants with the reference system needed during naïve learning walks. However, although not being necessary for the accuracy of the look-back behavior, we show that proper perception of the natural polarization pattern that changes over the day is important for triggering neuroanatomical changes in the CX and MB calyx that take place during learning walks. In the MB-calyx Co, this volume increase is linked to an increase in the number of MG synaptic complexes indicating that plasticity related processes are triggered when the ants are confronted with a naturally perceived polarization pattern that changes over the day.

ETHICS STATEMENT

This study was carried out in accordance with the Greek and German laws.

AUTHOR CONTRIBUTIONS

RG, PNF, RW, WR conceived the study. WR and RW led the study. RG, PNF and KG collected and analyzed the data. RG and PNF drafted and WR and RW revised the manuscript. All authors approved the final version of the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2017.00226/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

The role of celestial compass information in *Cataglyphis* ants during learning walks and for neuroplasticity in the central complex and mushroom bodies

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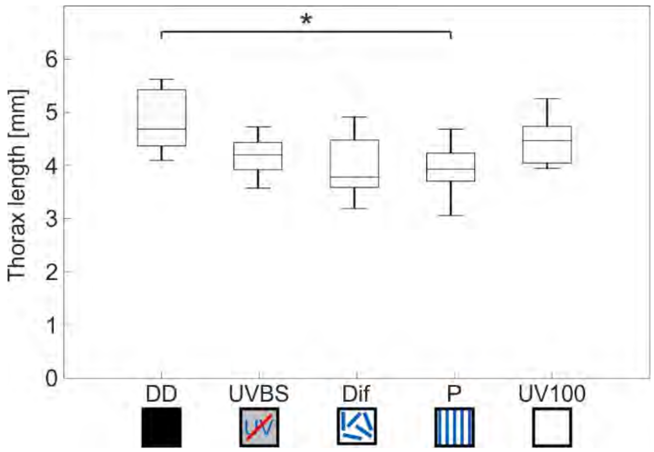
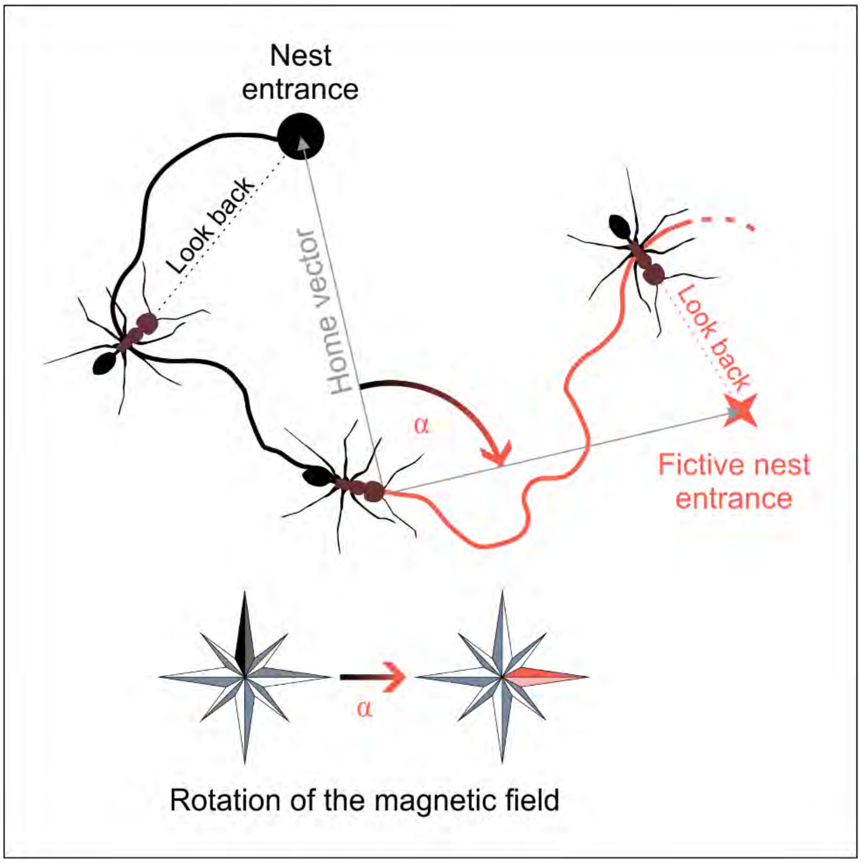
1 Supplementary Figure



Supplementary Figure 1. Comparison of the thorax length between the experimental groups. The central line of each boxplot depicts the median of the data. The upper and lower limits of the boxes show the 25th and 75th percentiles, while the whiskers extend to the extreme data points without outliers. A difference between the groups can be found using a Kruskal–Wallis test (χ_4^2=11.62; n=49; p=0.0240). The data was post-hoc compared to the DD group using the Mann-Whitney U-test with a Bonferroni correction. The asterisk indicates that data is significantly different (after correction $p<0.0125$) from the DD group. Only between DD and P the thorax length significantly differed (Mann-Whitney U-test with a Bonferroni correction: DD vs. P, Z_3=2.5404; n_{DD}=7; n_P=9; p=0.0118). No difference was found between the other groups compared to DD (DD vs. UVBS, Z_1=2.2642; n_{DD}=7; n_{UVBS}=11; p=0.0260; DD vs. Dif, Z_2= 2.1170; n_{DD}=7; n_{Dif}=9; p= 0.0418; DD vs. UV100, Z_4=2.5404; n_{DD}=7; n_{UV100}=13; p= 0.1779). Since no correlation between thorax length and the volume of the neuropils of interest was found, no correction for the allometric differences was used for the further analyses.

3 Manuscript 2: The Geomagnetic Field Is a Compass Cue in *Cataglyphis* Ant Navigation



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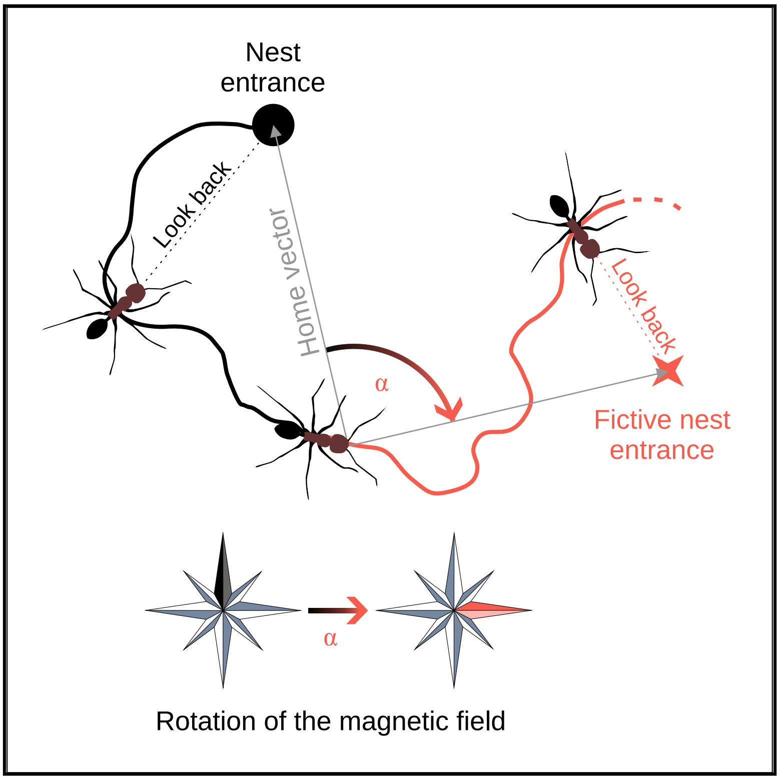
†Shared senior authorship.

Current Biology

Report

The Geomagnetic Field Is a Compass Cue in *Cataglyphis* Ant Navigation

Graphical Abstract



Highlights

- Desert ants frequently and robustly look back to the nest during learning walks
- To do so, the ants need to use information provided by their path integrator
- Novices use the geomagnetic field as a compass cue to determine the direction
- The geomagnetic field is the sufficient and necessary reference system for ants

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In Brief

Cataglyphis ant novices perform learning walks to acquire all information that is necessary for successful navigation as foragers later on. For that reason, they take snapshots of the nest's surrounding during pirouettes. Fleischmann et al. show that the geomagnetic field is necessary and sufficient for aligning the gazes to the invisible nest entrance.

Fleischmann et al., 2018, Current Biology *28*, 1440–1444
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Current Biology
Report

The Geomagnetic Field Is a Compass Cue in *Cataglyphis* Ant Navigation

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SUMMARY

Desert ants (*Cataglyphis*) are famous insect navigators. During their foraging lives, the ants leave their underground colonies for long distances and return to their starting point with fair accuracy [1, 2]. Their incessantly running path integrator provides them with a continually updated home vector [3–5]. Directional input to their path integrator is provided by a visual compass based on celestial cues [6, 7]. However, as path integration is prone to cumulative errors, the ants additionally employ landmark guidance routines [8–11]. At the start of their foraging lives, they acquire the necessary landmark information by performing well-structured learning walks [12, 13], including turns about their vertical body axes [14]. When *Cataglyphis noda* performs these pirouettes, it always gazes at the nest entrance during the longest of several short stopping phases [14]. As the small nest entrance is not visible, the ants can adjust their gaze direction only by reading out their path integrator. However, recent experiments have shown that, for adjusting the goal-centered gaze directions during learning walks, skylight cues are not required [15]. A most promising remaining compass cue is the geomagnetic field, which is used for orientation in one way or the other by a variety of animal species [16–25]. Here, we show that the gaze directions during the look-back-to-the-nest behavior change in a predictable way to alterations of the horizontal component of the magnetic field. This is the first demonstration that, in insects, a geomagnetic compass cue is both necessary and sufficient for accomplishing a well-defined navigational task.

RESULTS AND DISCUSSION

A first indication that the ants actually use the geomagnetic field as a compass cue came from a rather straightforward experiment in which we completely disarrayed the geomagnetic field by installing an electromagnetic spiral around the nest entrance. *Cataglyphis noda* ants most likely take nest-centered snapshots during the longest stopping phase of their pirouettes. With the spiral unpowered, the pirouetting ants correctly adjusted their gaze direction toward the nest entrance (Figure 1A), but when the spiral was powered, they no longer succeeded in gazing at the nest entrance and were oriented in random directions (Figure 1B; for statistical details in these and all following cases, see STAR Methods, "Statistics" section of Quantification and Statistical Analysis). To confirm this conclusion and to generate precisely controlled magnetic field situations, we next set up a Helmholtz coil system with the artificial nest entrance located in a platform at its center (Figure 2A). In all experiments performed under these conditions, the ants' gaze directions were analyzed during the first pirouette performed on the platform (control test, Figures 3A and 3C) and during the first pirouette after the Helmholtz coil had been turned on, i.e., after current had started to be passed through the two coils. The ants maintained their nest-centered gaze directions when the horizontal component of the earth's magnetic field was doubled in strength (Figure 3B), but when it was zeroed, the animals no longer selected a significant gaze direction (Figure 3D). As all celestial cues had remained unchanged, we conclude that the horizontal component of the geomagnetic field provides the necessary compass reference used by the ants for adjusting their viewing directions during their early learning walks.

If this conclusion is correct, rotating the horizontal component of the magnetic field should likewise lead to rotations of the ants' home vector and thus should shift the ants' gaze directions from the real to the fictive position of the nest entrance (Figure 2B, and Video S1). This was indeed the case. Irrespective of whether the horizontal component of the magnetic field was rotated through 90°, 180°, or −90° relative to its natural orientation, in their next occurring pirouette, the ants highly significantly gazed at the fictive rather than the real position of the nest entrance (compare Figures 4B', 4D', and 4F' with Figures 4B, 4D, and 4F). Due to the limited size of the platform necessary to ensure the ants' exposure to a homogeneous magnetic field between the two coils, the ant's spatial behavior following a pirouette under altered magnetic conditions could not be studied, as the ants frequently exited the experimental platform. Before the Helmholtz coil had been turned on, the very same ants had still correctly determined the true nest direction (Figures 4A, 4C, and 4E). Taken together,

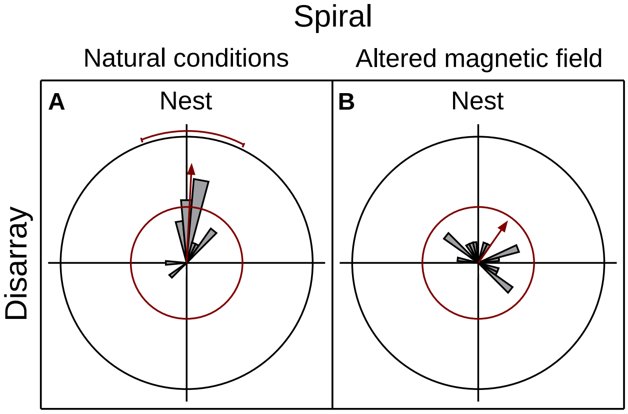

Figure 1. Effect of Disarraying the Magnetic Field with an Electromagnetic Spiral

(A) Natural conditions, i.e., electromagnetic spiral off (n = 15).

(B) Altered magnetic field, i.e., electromagnetic spiral on (n = 15). Data show the gaze directions during the longest stopping phase of pirouettes relative to the nest entrance (labeled "nest"). Data are shown in gray: The bins comprise 10°, and the circular axis has a maximal scale of 6 ticks, i.e., the outer black circle comprises 6 ants. Statistics are shown in red: The arrow indicates the r-vector, which is significantly directed if it exceeds the circle indicating the significance level of the Rayleigh uniformity test ($\alpha = 0.05$). If data are directed, the arc indicates the 95% confidence interval. For statistical details, see STAR Methods, section "Statistics."

these results provide clear evidence that at the transition from their indoor nursing to their outdoor foraging career, the ants employ a magnetic compass based on the horizontal component of the earth's magnetic field and do not rely on any other compass cue.

This completely unexpected discovery of a clear-cut magnetic compass in *Cataglyphis* ants raises at least two main questions. First, why do the ants employ a magnetic compass early in outdoor life, and second, whether, when, and how do they switch from the use of a magnetic compass to that of the visual sky compass, which has been shown to operate throughout the *Cataglyphis* ants' later foraging careers [6, 7]? As to the first question, one may argue that the earth's magnetic field could provide the ants with a directional reference already during the early stages of their lives, when while pursuing their underground nursing careers, they negotiate their ways through a labyrinth of subterranean gangways and tunnels. In this situation, idiothetic orientation might well be complemented by a geostable magnetic compass reference. This reference could even be used for path integration in the dark, as proposed for blind mole rats, which spend all their lives underground [26]. When the ants later start their outdoor activities and perform their learning walks upon first appearance above ground, they could well maintain their then-familiar geomagnetic compass reference for the following: (1) acquiring goal centered panoramic views and (2) calibrating their solar ephemeris function, i.e., correlating the sun's azimuthal position relative to earthbound coordinates with the time of day. As the present results show, the ants most likely accomplish the former task, but whether the geomagnetic field is also involved in the latter task cannot be decided yet. Rather, a suite of elegant experiments performed in honeybees support the hypothesis that the local

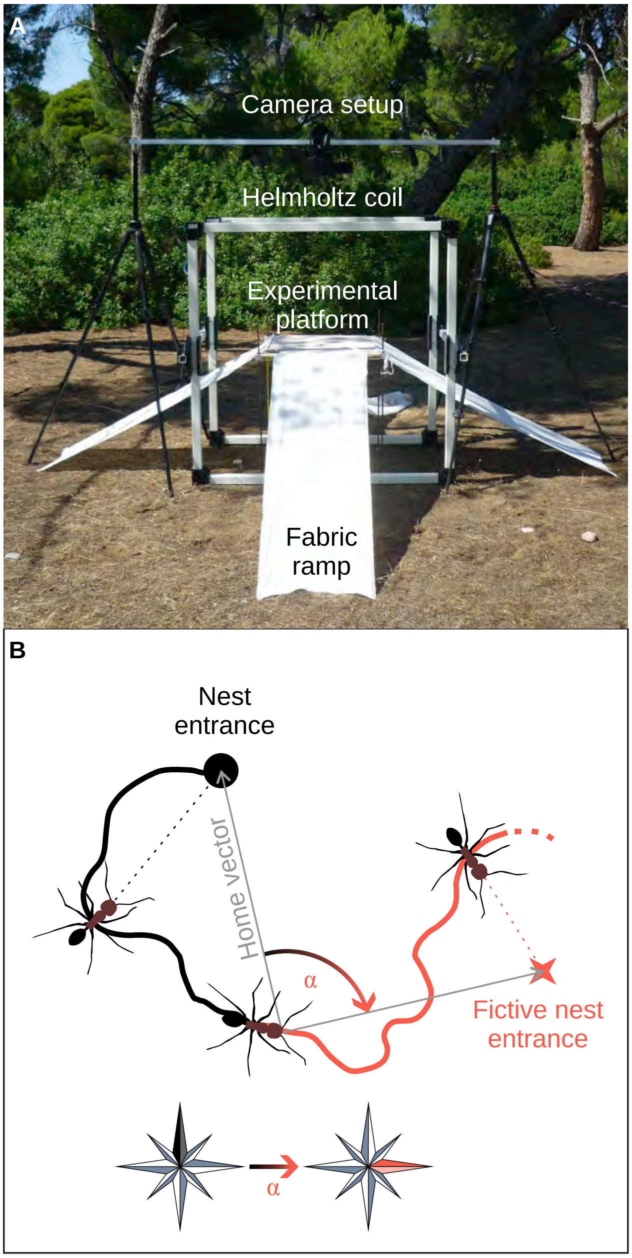

Figure 2. Experimental Settings

(A) Experimental setup (Helmholtz coil system, experimental platform, camera setup, fabric ramps) in the Greek pine forest.

(B) Proposed integration of magnetic information into the path integrator during a learning walk after rotation of the horizontal component of the magnetic field. The black line represents the ant's path during a learning walk before the magnetic field is altered. Its gaze direction during the longest stopping phase of a pirouette (dotted line) is directed toward the nest entrance (black dot). When the horizontal component of the magnetic field is rotated (in the example: $\alpha = 90°$), the home vector of the ant is rotated, as well. The ant continues its path (now shown in red) and eventually performs another pirouette. During the longest stopping phase, the ant now looks to the fictive nest entrance (indicated by the red star).

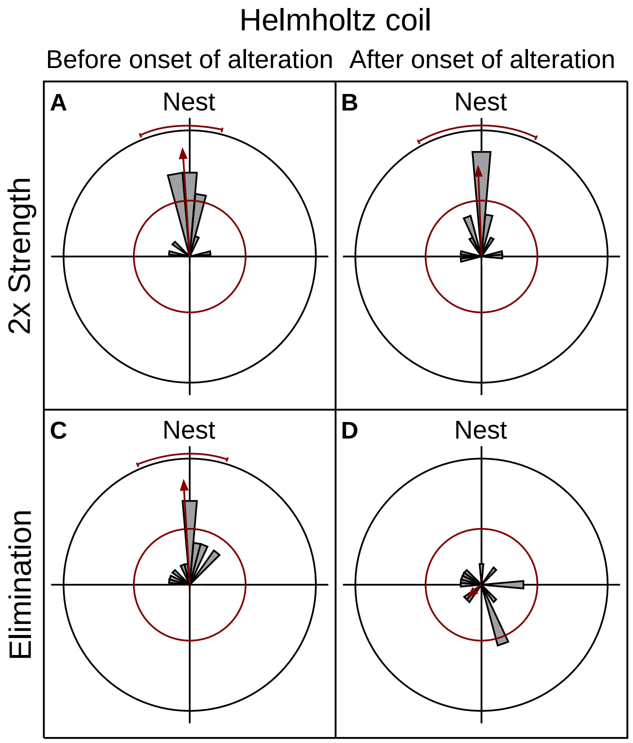

Figure 3. Effects of Strengthening and Elimination of the Horizontal Magnetic Field Component on Gaze Directions

(A and B) First pirouette before ([A] geomagnetic field, i.e., Helmholtz coil off) and first pirouette after ([B] double magnetic field strength, i.e., Helmholtz coil on) doubling the field strength of the horizontal component of the geomagnetic field during one learning walk (n = 15).

(C and D) First pirouette before ([C] geomagnetic field, i.e., Helmholtz coil off) and first pirouette after ([D] eliminated horizontal field component, i.e., Helmholtz coil on) elimination of the horizontal component of the geomagnetic field during one learning walk (n = 15). For explanations of data and statistics see Figure 1, and for statistical details, see STAR Methods, section "Statistics."

landmark skyline provides the decisive geostable reference for calibrating the solar ephemeris [27]. In birds, cross-calibration has been suggested between the geomagnetic field and celestial cues [28]. The learning walks of *Cataglyphis* ants provide an example that a magnetic compass may not only be important for long-distance navigation but might also be beneficial during short-range navigation [29].

The second question raised above sounds especially promising. As it is well established that, during their wide-ranging foraging journeys, *Cataglyphis* ants strongly rely on celestial compass cues [6, 7], on the one hand, one could expect that after the ants have finished their learning walk routine, they switch from one compass system to another. On the other hand, the ants might not switch at all but continue to use the geomagnetic field as a compass cue in particular navigational tasks such as acquiring goal centered panoramic views at particular locations. This hypothesis could be tested best by inducing fully fledged foragers to perform what we have called "re-learning walks." Such walks always occur after ants returning from a foraging journey have been experimentally confronted with a new landmark setting around the nest entrance. Thereafter, before starting their next foraging trip, the ants again exhibit their characteristic pirouetting behavior just as they have done previously in the beginning of their outdoor activities [30]. Do these ants, which by now have become experienced foragers, employ a magnetic compass in the way shown in the present account? Moreover, do they stick to this routine even when acquiring landmark views far afield in their foraging terrain? A hint that the latter could be the case comes from some early experiments in honeybees [20, 31]. Under certain conditions, bees seemed to use magnetic cues for visual landmark learning either to find a particular site defined by a single landmark or to distinguish between artificial panoramic patterns that differed only by their compass orientation. However, in all these cases, magnetic cues came into play, if at all [31], only when other cues were not available.

The same conclusion can be drawn from the few studies in which some other species of ants have been shown to respond, at least weakly and in particular contexts, to manipulations of the geomagnetic field [21–25]. Usually, as in weaver ants and leafcutter ants, foragers marching on trails could be deflected to some degree from their courses when the geomagnetic field was altered, but this happened only in particular instances, often only under sunless conditions [23], and sometimes not at all [24]; or the response markedly decreased when skylight cues became available [22]. All we can conclude from these studies is that when foraging ants are on their way, the earth's magnetic field might play some auxiliary compass role. In contrast, the present study provides unequivocal evidence that *Cataglyphis* ants, which also have been shown to treat a bar magnet as a beacon [25], rely exclusively on a magnetic compass while performing a well-defined navigational routine (adjusting goal-centered gaze directions during learning walks at the beginning of their outdoor lives). The ants do so even when geomagnetic cues are set in competition with celestial cues and with all other environmental cues available to them. In conclusion, when *Cataglyphis* ants turn back and look at the starting point of their learning walks, the nest entrance, and thus most likely acquire nest-centered views of the landmark surroundings, they use the horizontal component of the earth's magnetic field as the decisive (necessary and sufficient) compass cue.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
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- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - ○ Camera setup
  - ○ Experimental setup and procedure
  - ○ Electromagnetic spiral
  - ○ Helmholtz coil system
  - ○ Physical background of magnetic alterations
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - ○ Data analysis
  - ○ Statistics

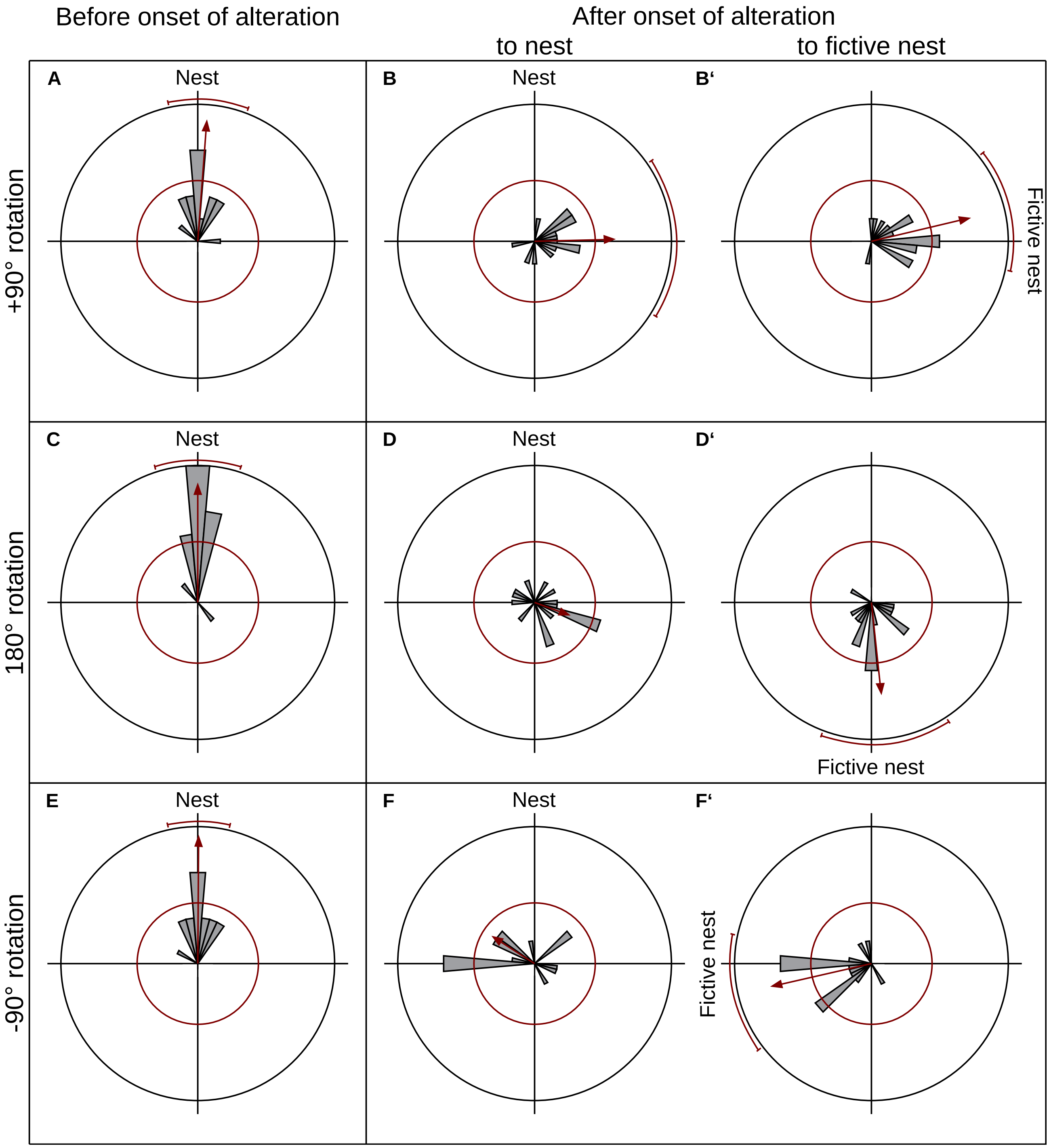

Figure 4. Rotation of the Horizontal Component of the Magnetic Field Predictably Changes Gaze Directions toward Fictive Nest Entrance Position

(A–F) Gaze directions of first pirouettes before ([A, C, and E] geomagnetic field, i.e., Helmholtz coil off) and of first pirouettes after ([B] +90°; [D] 180°; [F] −90° rotated horizontal component of magnetic field, i.e., Helmholtz coil on) rotation during one learning walk (n = 15 for each rotation) relative to the nest entrance. (B', D', F') Same data as in (B), (D), and (F) relative to fictive nest entrance position. For explanations of data and statistics see Figure 1, and for statistical details, see STAR Methods, section "Statistics," for the necessity to calculate the gaze directions relative to both expectancy values (actual and the fictive nest entrance), see Figure 2B.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one video and can be found with this article online at https://doi.org/10.1016/j.cub.2018.03.043.

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AUTHOR CONTRIBUTIONS

P.N.F, R.G., and V.L.M. came up with the initial idea and design for the spiral experiment. All authors conceived the Helmholtz Coil experiment. W.R. and R.W. led the study, and W.R. was responsible for funding acquisition. P.N.F. and R.G. conducted the experiments, and V.L.M. helped to set them up and made all calculations necessary. P.N.F. and R.G. analyzed the data and prepared figures and Video S1. All authors discussed the results. P.N.F. and R.G. drafted a first version and R.W. drafted a second version of the manuscript. All authors revised the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Cataglyphis noda (Brullé 1832)	Schinias National Park, Marathonas, Greece	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pauline Nikola Fleischmann (pauline.fleischmann@uni-wuerzburg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Two ant colonies of *Cataglyphis noda* (Brullé 1832) located in different clearings in the pine forest of Schinias National Park, Marathonas, Greece (38°08'N 24°01'E; geomagnetic field strength (46.1 ± 0.2) μT; horizontal component (26.6 ± 0.2) μT (https://www.ngdc.noaa.gov/geomag-web/)) were used for the experiments during summers of 2016 (spiral experiment) and 2017 (Helmholtz coil experiment). Before the experiments started all ants outside the nest were marked with car paint (Motip Lackstift Acryl, MOTIP DUPLI GmbH, Haßmersheim, Germany) for three consecutive days to ensure that only unmarked novices performing naive learning walks were included in the experiments.

METHOD DETAILS

Camera setup

Above the experimental area (i.e., above the natural nest entrance in the spiral experiment, or above the experimental table in the Helmholtz coil experiment, Figure 2A) two cameras were installed. A camcorder (HDR-CX330E, Sony Corporation, Minato, Japan) recorded the experimental area nonstop during the experiments at 25fps. A 4K-camcoder (HC-X1000, Panasonic Corporation, Kadoma, Japan) recorded learning walks of novices at 50fps. Every time an unmarked ant left the nest entrance, the observer sitting next to the experimental setup started the recording of the 4K-camcoder using the Panasonic Image App (Version 10.9.2, Panasonic Corporation, Kadoma, Japan) on a Cat S60 smartphone (Caterpillar, Peoria, USA or a Xperia Z1, Sony, Tokyo, Japan). High-speed recordings were stopped when the ant returned to the nest or left the experimental platform (60cm x 60cm), i.e., fell off the platform or walked down one of the fabric ramps.

Experimental setup and procedure

After three days of marking ants outside the nest, experimental trials were conducted for maximally three consecutive days, i.e., the learning walks performed were most likely early learning walks of novices [12–14].

Electromagnetic spiral

To disarray the magnetic field at the natural nest entrance a flat coil (diameter 70cm, separation distance between the wire windings: 5cm; diameter of copper wire: 1mm) was installed around the nest entrance and covered with sand. The electromagnetic spiral was powered with three 4.5 Volt batteries (3R12, VARTA Consumer Batteries GmbH & Co. KGaA, Ellwangen, Germany) wired in parallel to confront ants with a radial magnetic field slightly stronger than the earth's magnetic field close to the ground. This altered magnetic field provided different directional information at any point on the spiral. Therefore, it was not suitable as a reliable reference system for the looks back to the nest entrance during learning walks. As a control, ants were recorded during learning walks when the spiral was switched off.

Helmholtz coil system

To confront ants with a precise magnetic field (controlled direction and strength) a Helmholtz coil system was used (HHS 5213-100, Schwarzbeck Mess-Elektronik, Schönau, Germany). Current was supplied to the two coils with a customized DC power supply made by the Biocenter's electronic workshop. The current was constantly monitored with a multimeter (VC820-1, Voltcraft, Hirschau, Germany). Since homogeneity of the magnetic field is highest in the center of the coil system, ants had to perform their learning walks on a platform (60cm x 60cm, Figure 2A). For that reason, the natural nest entrance was covered with a cylindrical box (nest cover) with a tunnel (diameter: 3cm) so that the ants could still leave the nest but had to use an artificial nest entrance. The Helmholtz coil system and the camera setup were installed every morning and removed every evening. During the experiment, the tunnel of the nest cover was connected with the experimental table via a flexible tube (diameter: 3cm). Ants left the tube through a hole (diameter: 3cm) in the center of the elevated platform. They could leave the platform by walking on one of four fabric ramps (Figure 2A). Foragers learned quickly to use these ramps for outbound and inbound trips. When a novice performed a naive learning walk, the Helmholtz coil was switched on after the ant had performed at least one pirouette. The recording was stopped, when the ant returned to the nest entrance or left the platform. Then the Helmholtz coil was also switched off.

Physical background of magnetic alterations

The geomagnetic field can be altered by inducing an additional magnetic field with suitable current distributions. Once the current distribution is known, this additional magnetic field can be calculated with the Biot-Savart law. According to the superposition principle, the combined magnetic field is then found by vector addition of the two contributions.

In the case of the spiral, a flat coil setup, the resulting magnetic field is approximately radial in the plane above the spiral and mostly vertical in between the windings. On the whole, the total magnetic field is changing rapidly from one point to another which makes it unsuitable as a reference system for the ants. The current through the spiral was chosen to be slightly above the threshold for inducing movement of a magnetic compass needle in close proximity to the spiral. This procedure, together with a rough numerical estimation, ensured that the artificial field strength was in the same order of magnitude as the natural field strength.

The Helmholtz coil on the contrary generates a very homogeneous magnetic field, which can still be used as a reference system. For reinforcement, elimination, and 180° rotation experiments, the coil axis was aligned parallel to the horizontal component of the natural magnetic field with the aid of a magnetic compass (Fluorescent Map Compass 3116, AceCamp GmbH, Offenbach, Germany). For the +90° and −90° rotation experiments, the coil axis was horizontally rotated about +45° and −45° with respect to the horizontal component of the natural magnetic field.

The Helmholtz coil used in the experiments had a rectangular cross-section with an electrical side length of 1.30 m. The coil separation was 0.71 m, which offers best uniformity along an axis through the coil centers. The systematic error in field strength on the experimental platform (60cm x 60cm in 71cm height) due to remaining field inhomogeneity in the Helmholtz coil was less than 10%. In the reinforcement, elimination, and 180° rotation experiments, this results in a field strength variation of similar order. In the 90° rotation experiments, this corresponds to a directional variation of the total magnetic field of less than 4°. Compared to the spatial variations, the uncertainty in magnetic field strength due to the current measurement and coil alignment is negligible.

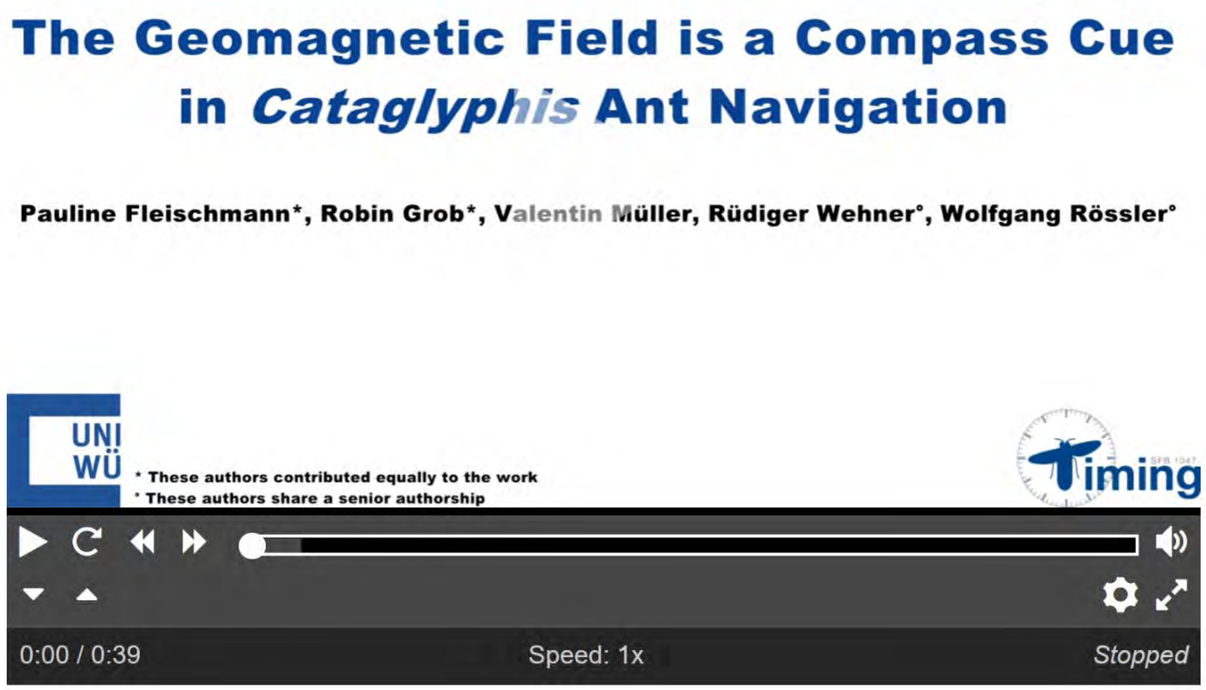
QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis

The 4K-videos were converted into image stacks using the Free Video to JPG Converter (v. 5.0.99 build 823, DVDVideoSoft, DIGITAL WAVE LTD., London, UK). Pirouettes (full or partial tight turns about the ant's body axis [14]) were analyzed manually frame by frame using the MATLAB (2015a, MathWorks, Natick, MA, USA) application DIGILITE (Jan Hemmi and Robert Parker, The Australian National University, Canberra, Australia). The positions of thorax and mandibles were marked in each frame. In addition, the nest entrance was marked. Using these coordinates the gaze directions of the ants could be determined relative to the nest. The direction of the nest was defined as 180°. Stopping phases during pirouettes were defined as it was done before (minimal duration: 100ms) [14, 15]. The longest stopping phase of each pirouette was used to compare gaze directions between experimental groups. In the spiral experiment, 15 pirouettes were analyzed when the spiral was on and 15 pirouettes when the spiral was off. In all other experiments both the first pirouette an ant performed on the experimental platform and the first pirouette after the Helmholtz coil had been switched on were analyzed (n = 15 per experiment). In the experiments where the magnetic field was rotated (+90°, 180° and −90°, respectively) the fictive nest entrance position was calculated. Since it was different for every test ant (Figure 2B), it was determined individually for every ant. Data of the pirouette after the Helmholtz coil was switched on were analyzed twice – once relative to the nest entrance and once relative to the fictive nest entrance position.

Statistics

Circular statistics were performed using Oriana 4.02 (Kovach Computing Services, Anglesey, UK). Gaze directions during the longest stopping phases of pirouettes were grouped into 10°-bins. To check whether data were randomly distributed or directed, the Rayleigh test was used ($\alpha = 0.05$) (Rayleigh Uniformity Test and Mean Vector (μ): Figure 1A: $Z_0 = 8.934$, $n = 15$, $p < 0.001$, $\mu = 182.8$; Figure 1B: $Z_0 = 2.296$, $n = 15$, $p = 0.099$, $\mu = 215.2°$; Figure 3A: $Z_0 = 10.855$, $n = 15$, $p < 0.001$, $\mu = 176.1°$; Figure 3B: $Z_0 = 7.525$, $n = 15$, $p < 0.001$, $\mu = 177.9°$; Figure 3C: $Z_0 = 10.102$, $n = 15$, $p < 0.001$, $\mu = 176.6°$; Figure 3D: $Z_0 = 0.283$, $n = 15$, $p = 0.283$, $\mu = 47.8°$; Figure 4A: $Z_0 = 11.531$, $n = 15$, $p < 0.001$, $\mu = 184.4°$; Figure 4B: $Z_0 = 5.019$, $n = 15$, $p = 0.005$, $\mu = 268.5°$; Figure 4B': $Z_0 = 8.038$, $n = 15$, $p < 0.001$, $\mu = 166.8°$; Figure 4C: $Z_0 = 11.100$, $n = 15$, $p < 0.001$, $\mu = 180.0°$; Figure 4D: $Z_0 = 1.051$, $n = 15$, $p = 0.356$, $\mu = 289.6°$; Figure 4D': $Z_0 = 6.672$, $n = 15$, $p < 0.001$, $\mu = 173.8°$; Figure 4E: $Z_0 = 12.839$, $n = 15$, $p < 0.001$, $\mu = 180.4°$; Figure 4F: $Z_0 = 1.942$, $n = 15$, $p = 0.144$; $\mu = 122.9°$; Figure 4F': $Z_0 = 8.271$, $n = 15$, $p < 0.001$, $\mu = 167.3°$). If data were directed, the 95% confidence intervals were determined to check whether the expected direction (nest entrance or fictive nest entrance defined as 180°) was within these limits (95% Confidence Interval (−/+): Figure 1A: 159.9°/205.7°; Figure 3A: 157.8°/194.3°; Figure 3B: 151.2°/204.6°; Figure 3C: 156.5°/196.7°; Figure 4A: 169.9°/200.9°; Figure 4B: 235.4°/301.5°; Figure 4B': 141.6°/192.1°; Figure 4C: 162.4°/197.7°; Figure 4D': 146.9°/200.7°; Figure 4E: 167.7°/193.1°; Figure 4F': 142.6°/191.9°).



The Geomagnetic Field is a Compass Cue in *Cataglyphis* Ant Navigation

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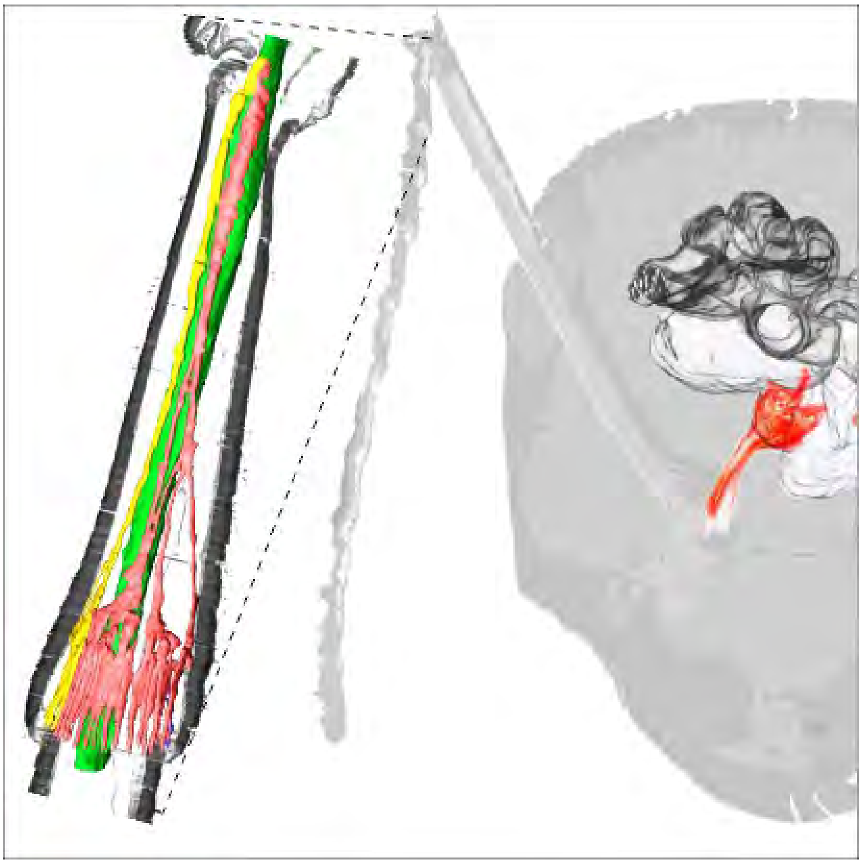
° These authors share a senior authorship

The video can be downloaded from:
https://ars.els-cdn.com/content/image/1-s2.0-S0960982218303725-mmc1.mp4

4 Manuscript 3: Johnston's Organ and its Central Projections in *Cataglyphis* Desert Ants



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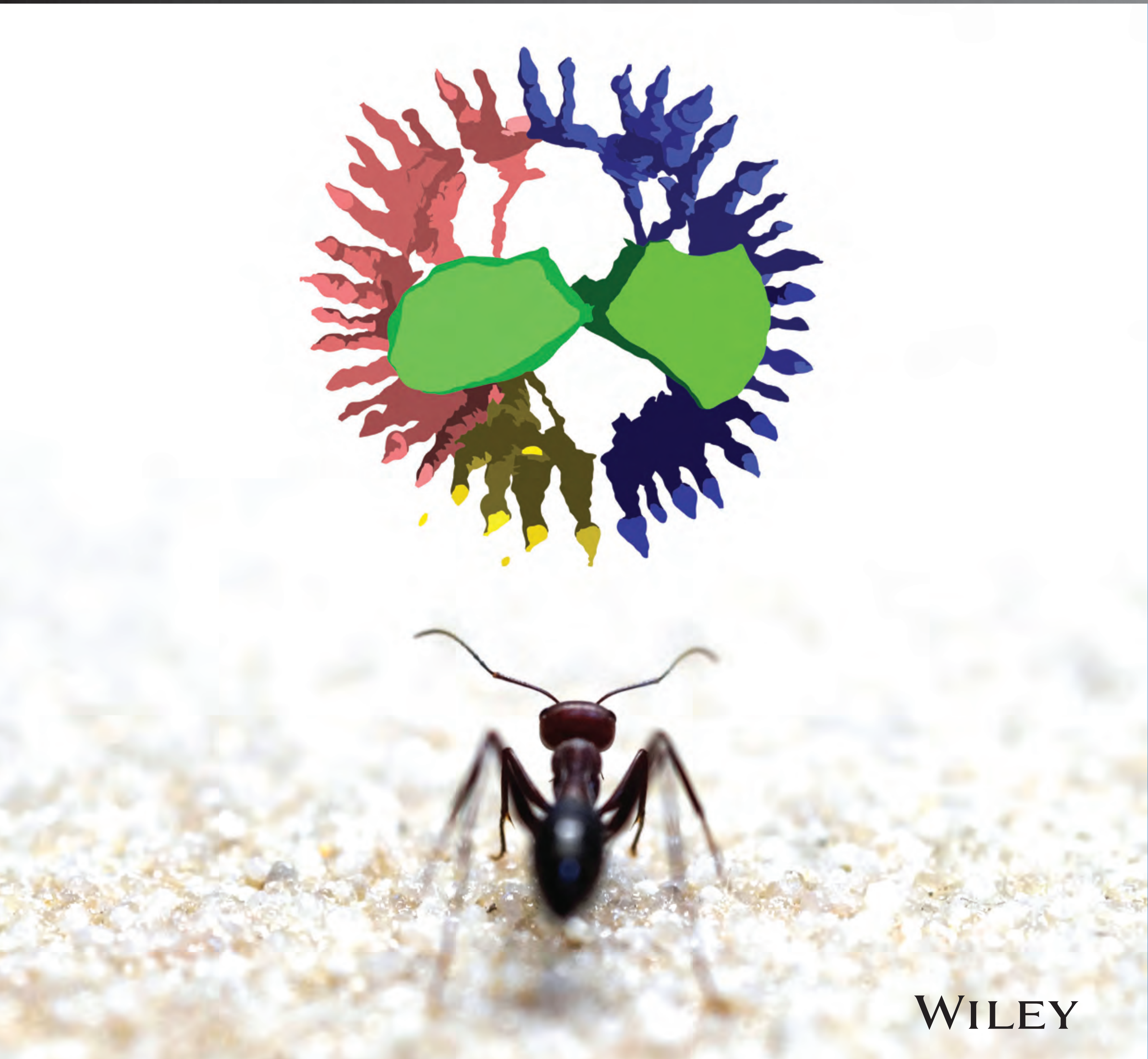

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RESEARCH ARTICLE

Johnston's organ and its central projections in *Cataglyphis* desert ants

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Abstract

The Johnston's organ (JO) in the insect antenna is a multisensory organ involved in several navigational tasks including wind-compass orientation, flight control, graviception, and, possibly, magnetoreception. Here we investigate the three dimensional anatomy of the JO and its neuronal projections into the brain of the desert ant *Cataglyphis*, a marvelous long-distance navigator. The JO of *C. nodus* workers consists of 40 scolopidia comprising three sensory neurons each. The numbers of scolopidia slightly vary between different sexes (female/male) and castes (worker/queen). Individual scolopidia attach to the intersegmental membrane between pedicel and flagellum of the antenna and line up in a ring-like organization. Three JO nerves project along the two antennal nerve branches into the brain. Anterograde double staining of the antennal afferents revealed that JO receptor neurons project to several distinct neuropils in the central brain. The T5 tract projects into the antennal mechanosensory and motor center (AMMC), while the T6 tract bypasses the AMMC via the saddle and forms collaterals terminating in the posterior slope (PS) (T6I), the ventral complex (T6II), and the ventrolateral protocerebrum (T6III). Double labeling of JO and ocellar afferents revealed that input from the JO and visual information from the ocelli converge in tight apposition in the PS. The general JO anatomy and its central projection patterns resemble situations in honeybees and *Drosophila*. The multisensory nature of the JO together with its projections to multisensory neuropils in the ant brain likely serves synchronization and calibration of different sensory modalities during the ontogeny of navigation in *Cataglyphis*.

KEYWORDS
ant brain, chordotonal organ, graviception, magnetic compass, multisensory integration, navigation, wind compass

1 | INTRODUCTION

Johnston's organ (JO) is a highly elaborated multisensory organ in the insect antenna. Being present in most insects, the JO shows a wide range in structural organization (Child, 1894; McIver, 1985). For example in flies the JO comprises ~720 sensory neurons in *Drosophila melanogaster* (Kamikouchi, Shimada, & Ito, 2006), but it may contain as many as 30,000 sensory neurons in mosquitos (Boo &

Richards, 1975). This diversity is connected to a multitude of functions (Child, 1894; McIver, 1985). The JO is known as a chordotonal organ, a mechanoreceptive organ sensitive to the slightest antennal movements (review: Yack, 2004). The antennal deflections may be caused by gravitational or wind forces (review: McIver, 1985). Therefore, the JO is involved in graviception (Kamikouchi et al., 2009; Vowles, 1954), flight control, and the detection of wind directions (Sane, Dieudonné, Willis, & Daniel, 2007). Johnston himself classified the JO as an auditory apparatus in *Culex* mosquitoes (Johnston, 1855). In the fruit fly *D. melanogaster* (Göpfert & Robert, 2001a; Göpfert & Robert, 2002) and the mosquito *Aedes aegypti* (Cator, Arthur, Harrington, & Hoy, 2009), the JO detects airborne sounds, for example, during courtship. In the honeybee *Apis mellifera*, the JO plays a role in detecting airborne and vibrational sounds produced during the waggle dance, a form of communication that informs nestmates about the distance and direction of food sources (Dreller & Kirchner, 1993a; Dreller & Kirchner, 1993b; Dreller & Kirchner, 1995).

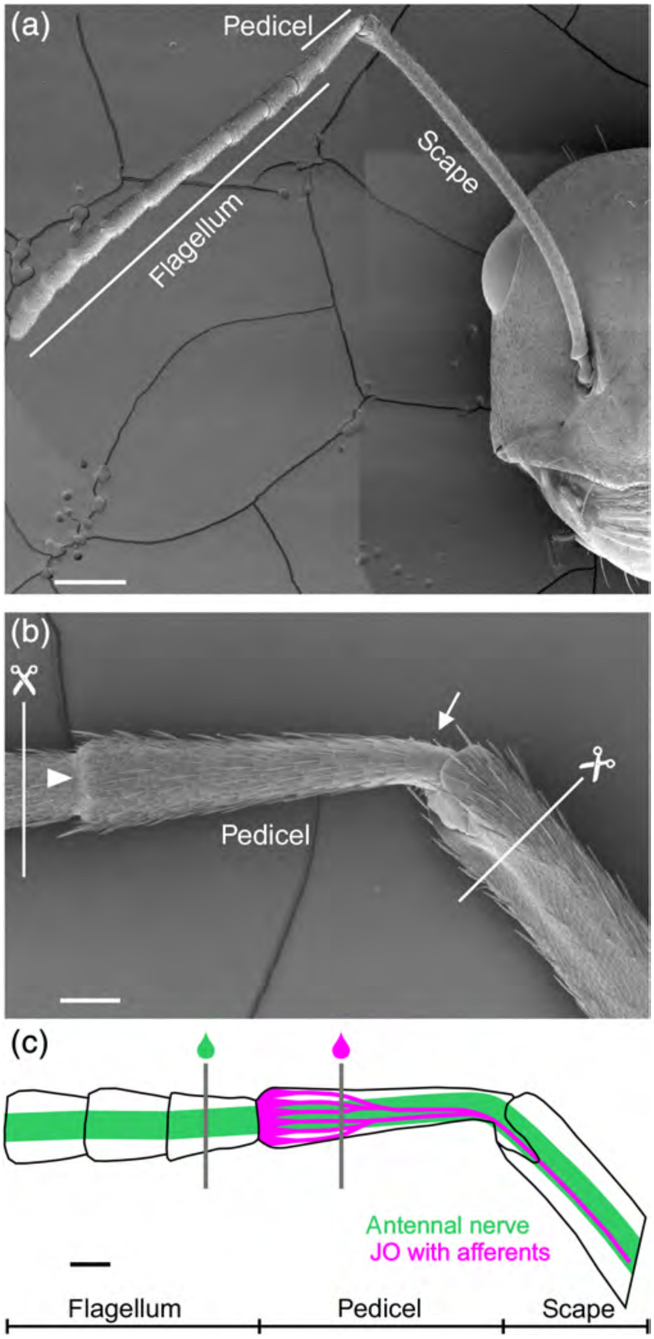

FIGURE 1 The *Cataglyphis nodus* antenna. (a) Scanning electron microscopy image of the ant's (*C. nodus* worker) head with the antenna. The antenna is divided into three parts: The flagellum is located most distally from the head. It comprises 10 flagellomeres. The middle part is the pedicel, which houses mechanosensory organs, particularly the Johnston's organ (JO). The scape is connected to the head capsule. Scale bar 500 μm. (b) Close up of the pedicel of a *C. nodus* worker. At the joint between flagellum and pedicel, dents in the cuticle indicate the attachment sites of the JO to the intersegmental membrane (arrowhead). A hair plate is located close to the joint of pedicel and scape (arrow). For serial sectioning, the antenna was cut (scissors symbols) at the first flagellomere and at an equivalent distance from the pedicel at the scape before fixation. Scale bar 100 μm. (c) Scheme of the anterograde double staining procedure. The antenna was first cut at the first flagellomere and dye was applied (green droplet). After 3 h, the antenna was cut at the level of the pedicel to apply a second dye (magenta droplet). This technique allowed for a differential staining of the antennal nerve without afferents of the JO (green) and with afferents of the JO (magenta). Scale bar 100 μm

The peripheral morphology of the JO in the antenna has been studied in detail in Diptera (e.g., fruitflies: Göpfert & Robert, 2002; mosquitos: Johnston, 1855), in Hymenoptera (e.g., Child, 1894; bees: Ai, Nishino, & Itoh, 2007; Dreller & Kirchner, 1995; sawflies: Hallberg, 1981; ants: Masson & Gabouriaut, 1973; Vowles, 1954), and many other insect species (e.g., Snodgrass, 1926). The sensory structures are located at the joint of the pedicel and the flagellum (Figure 1a) and attached to the intersegmental membrane. The JO consists of a species-specific number of mechanosensory complexes, so-called scolopidia. Individual scolopidia usually comprise four different cell types: (1) between one to four sensory neurons, (2) scolopale cells that ensheath the dendrites of the sensory neurons, (3) cap cells, and (4) glial cells—sometimes also referred to as supporting cells (review: Yack, 2004). Via the cap cells, the scolopidia attach to the intersegmental membrane by chitin caps. These attachment sites can be observed as pits on the outside of the pedicel's most distal end (Snodgrass, 1926). The caps are connected to scolopale rods within the scolopale cells, and the mechanosensitive portions of the dendrites are suspended within the rods (Todi, Sharma, & Eberl, 2004).

To detect antennal movement, Todi et al. (2004) suggested a "bow and string" mechanism for the function of the JO: Antennal movement deflects the intersegmental membrane between pedicel and flagellum. This force is transduced via the cap cells to the scolopale cells and the dendrites, on which mechanosensitive ion channels are opened. The resulting action potentials are transmitted along the JO afferent projections into the central brain (Todi et al., 2004).

Although the morphology of the JO has been studied in various insect species, the afferent projections of the JO came into focus in only a limited number of species. In the honeybee, mechanosensory information from the antennae projects into the antennal mechanosensory and motor center (AMMC) (e.g., Maronde, 1991). In both the honeybee and *Drosophila* sensory afferents of the JO project along the antennal nerve (AN) into the AMMC (Ai et al., 2007; Brockmann & Robinson, 2007; Kamikouchi et al., 2006). In *D. melanogaster*, different sensory modalities—like sound and wind—activate different populations of JO neurons. These innervate distinct areas within the AMMC (Yorozu et al., 2009). This enables the JO to function as an efficient multisensory organ that distinguishes between different sensory modalities and qualities. Additionally, the JO afferents project into the ventral region of the ventrolateral protocerebrum, and the posterior part of the subesophageal ganglion (*A. mellifera*: Ai et al., 2007; *D. melanogaster*: Kamikouchi et al., 2006). Interestingly, JO afferents terminate in similar brain areas like projections from visual input regions, that is, the compound eyes and the ocelli (Ai et al., 2007; Maronde, 1991; Okubo, Patella, D'Alessandro, & Wilson, 2020). The integration of compass information from both visual and JO input likely is important for successful multimodal navigation, especially in challenging habitats (Okubo et al., 2020).

Despite the fact that ants are highly tactile insects, surprisingly little research has been done on the fine structure of the JO in ants (*Camponotus vagus*: Masson & Gabouriaut, 1973; *Formica rufa*: Vowles, 1954), and none on the central projections of JO receptor neurons. One major difference between especially flies and ants is that the latter are not able to perceive airborne sound (Roces & Tautz, 2001). This, however, provides a unique opportunity to study the involvement of the JO in other sensory modalities than hearing.

Cataglyphis desert ants have been favorable experimental models for sensory orientation for decades, mainly because of their astonishing navigational capabilities and the involved sophisticated navigational tool kit (review: Wehner, 2003, 2020). *Cataglyphis* is intensively studied for the use of polarized skylight as directional information for path integration during homing back to their nest after far ranging food searches. Many of their navigational tools, like the skylight compass or panoramic landmark orientation, are based on vision and, therefore, the ants' eyes. However, for several navigational components the location of the sensory organ is still unknown or remained speculative: During early learning walks, *Cataglyphis nodus* uses the earth's magnetic field as a compass (Fleischmann, Grob, Müller, Wehner, & Rössler, 2018). Later, during foraging, *Cataglyphis* ants use gravitational information to account for slopes during path integration along uneven terrain (Ronacher, 2020; Wohlgemuth, Ronacher, & Wehner, 2001). To finally pinpoint food sources on their extensive foraging trips or the nest entrance in the final approach during homing, *Cataglyphis fortis* also utilizes a wind compass (Steck, Hansson, & Knaden, 2009; Wolf & Wehner, 2000). Especially for the latter two, the JO is a promising candidate for the responsible sensory organ, which already received support from experiments including antennal manipulations (Wolf & Wehner, 2000). As an interesting further aspect, in contrast to the ambulatory worker caste, the sexual castes of *Cataglyphis* (queens and males) are capable of flight (Peeters & Aron, 2017) and therefore require additional sensory machinery for flight stabilization. Surprisingly, until now the JO in the antenna of *Cataglyphis* ants had not been investigated in detail. In this study, we analyze the three dimensional structure of the JO in the antenna of *C. nodus* together with the central projections of its sensory afferents into the ant brain. Additionally, we compare the JO in young *C. nodus* queens and males with the JO in workers. This together provides first insight toward understanding the role of the antennae, particularly the JO, in providing various sensory channels feeding into brain neuropils potentially involved in navigational tasks in desert ants.

2 | MATERIAL AND METHODS

For the experiments, colonies of *Cataglyphis nodus* (Brullé 1832) were excavated in the pine forests of Schinias national park (38.153783, 24.030913) and Strofylia national park (38.155199, 21. 376582) in Greece during the summers of 2016 and 2019, and subsequently maintained in our ant facility at Zoology II, University of Würzburg. The colonies were housed in a climate chamber (ThermoTec Weilburg GmbH & Co. KG, Weilburg, Germany) at 29°C and 40% humidity. Nest boxes were placed in a dark cabinet that was connected to a foraging arena that offered a 12 h light 12 h dark circle. Adult workers were collected from these colonies in Würzburg. Virgin young queens and males were collected directly from ant colonies in Greece and transported to Würzburg. Ants were anesthetized on ice prior to the experiments.

2.1 | Scanning electron microscopy

For obtaining high-resolution images of the ant antennae, ants were cold-anesthetized and decapitated. Heads of workers, virgin queens, and males were fixated overnight in 6.25% glutaraldehyde in 50 mM phosphate buffer solution (pH 7.4) at 4°C on a shaker. The fixated heads were then rinsed five times for 5 min each in Sørensen phosphate buffer (pH 7.4) (Sörensen, 1912) before being dehydrated in an increasing acetone serial dilution in distilled water at 4°C on a shaker. The dehydration steps were: 15 min in 30%, 20 min in 50%, 30 min in 75%, 45 min in 90%, and twice for 30 min in 100% acetone in water solution. The ant heads were then placed inside a Critical Point Dryer (BAL-TEC CPD 030, BAL-TEC AG, Balzers, Liechtenstein) filled with 100% acetone and dried using CO_2. After dehydration, the heads were sputtered with gold–palladium using a Sputter Coater (BAL-TEC SCD 005, BAL-TEC AG, Balzers, Liechtenstein). The antennae and heads were scanned using a field emission scanning electron microscope (JSM-7500F, JEOL Ltd., Akishima, Japan).

2.2 | Sectioning and 3D reconstruction of the JO

We prepared serial sections of the pedicel to analyze the detailed structure of the JO in *C. nodus*. Decapitated ant heads of workers, virgin queens, and males were fixated in wax, and the antennae were cut in the middle of the first flagellomere behind the pedicel and at equal distance from the pedicel at the scape (Figure 1b). For further preparation, the pedicels were placed in small baskets with a glass fiber mesh (MN 85/70–403007, Macherey-Nagel GmbH & Co. KG, Düren, Germany) at the bottom. After dissection, the pedicels were immediately fixated in ice-cold 1.5% formaldehyde, 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.7) with 0.04% $CaCl_2$ on a shaker overnight at 4°C. Subsequently, the antennal parts were rinsed with 30% ethanol in water solution and then dehydrated in an increasing ethanol serial dilution for 30 min in each step: 50%, 70%, 90%, 95%, and twice in 100% ethanol in water solution. The pedicels were cleared twice for 10 min in propylene oxide. Each pedicel was subsequently embedded in Epon 812 (Epon 812, SERVA Electrophoresis GmbH, Heidelberg, Germany) by using an Epon 812 in propylene oxide serial dilution for at least 4 h in each step: 25%, 50%, 75%, and 100% Epon 812 in propylene oxide solution. Subsequently, the pedicels were mounted in 100% Epon 812 in small silicon molds and heated at 60°C for 72 h.

For light microscopy, the embedded pedicels were cut in serial sections of 1.5 μm (cross sections) or 1.4 μm (longitudinal sections) thickness using an ultramicrotome (Leica EM UC7, Leica Microsystems GmbH, Wetzlar, Germany) and stained for contrast using 1% azure II, 1% methylene blue, and 1% borax in water for 1–2 min at 60°C. The stained serial sections were then mounted using Epon 812 on glass slides, covered with a thin coverslip (Cover Slips, Thickness 0, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and hardened for 24 h at 60°C.

The serial sections were imaged using a light microscope (Axiophot, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a digital camera (VisiCAM-100, Visitron Systems GmbH, Puchheim, Germany) using VisiView 2.1.4 (Visitron Systems GmbH, Puchheim, Germany). The images from serial sections were aligned using the TrackEM2 (Cardona et al., 2012) plugin for ImageJ 1.52n (Wayne Rasband, National Institutes of Health, USA) and 3D reconstructed in Amira (Amira-Avizo Software 2019.1, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

2.3 | Anterograde tracings of JO afferents and 3D reconstruction

To label the afferents of sensory neurons of the JO in the pedicel of *C. nodus*, a double anterograde staining procedure was performed using a combination of techniques previously applied in the honeybee (e.g., double staining (Kirschner et al., 2006) and bilateral staining (Ai et al., 2007)) and *Cataglyphis* (Habenstein, Amini, Grübel, el Jundi, & Rössler, 2020). The head of cold-anesthetized ants was fixed with dental wax. The antenna was cut at the first flagellomere and a Dextran, Alexa Fluor 488 (D22910, Life Technologies GmbH, Darmstadt, Germany) droplet was placed on the cut end of the antennal nerves (Figure 1c). During incubation for 3–4 h at room temperature in a dark box with high humidity, the dye was allowed to be transported along the antennal nerve branches into the ant's brain. Subsequently, the antenna was cut further proximally at the pedicel and a dextran tetramethylrhodamine droplet (micro-Ruby, D-7162, Life Technologies GmbH, Darmstadt, Germany) was placed on the cut end (Figure 1c). This allowed for specific staining of the central projections of the JO afferents, since projections of the more distal antennae were already stained with Dextran, Alexa Fluor 488, and farther proximal projections (e.g. from the bristle fields at the base of the pedicel) remained excluded. The dye was again incubated for 3–4 h at room temperature in a dark box with high humidity. Afterwards, a small window was cut into the head capsule of the ants to dissect the brain under cooled ant ringer solution (127 mM NaCl, 7 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.8 mM TES, and 3.2 mM trehalose, pH 7.0). The brains were fixated overnight in 4% formaldehyde in phosphate-buffered saline (PBS) solution. Afterwards, the brains were rinsed three times for 10 min in PBS and dehydrated using an ethanol in water serial dilution with 10 min in each step: 30%, 50%, 70%, 90%, and twice in 100% ethanol. The dehydrated brains were then cleared in methyl salicylate (4529.1, Carl Roth Gmbh & Co. Kg, Karlsruhe, Germany). This technique stained specifically for the JO afferents with the second tracer. Other, non-JO afferents from the antennae will be labeled with both tracers. Since the first tracer, however, is applied earlier to the afferents from the flagellum, it will have twice the time to be transported. Thus, the staining with the first tracer (green) will appear more prominently in the merged images.

To trace the projections of the complete antennal nerve labeled at the level of the pedicel in distinct neuropils of the ant brain and compare them with double stained preparations (see above), we used combined immunostaining with anti-synapsin antibodies. The antenna was cut at the pedicel and stained with Dextran tetramethylrhodamine only. Subsequently, the brains were dissected and fixated overnight at 4°C in a 4% formaldehyde in water solution. The next day, the brains were rinsed three times for 10 min each in PBS before being rinsed once in 2% Triton-X 100 solution in PBS and twice in 0.5% Triton-X 100 solution in PBS, for 10 min each, to permeabilize cell membranes for antibody application on whole mount brains. The brains were subsequently incubated for 1 h at room temperature on a shaker in a 0.5% Triton-X 100 in PBS with 2% of Normal Goat Serum (NGS, Jackson ImmunoResearch Laboratories, West Grove, USA) solution to block unspecific binding sites. To label synapse-rich neuropils, the brains were then incubated for 3 days at 4°C on a shaker with the primary anti-synapsin antibody from mouse (SYNORF1, kindly provided by E. Buchner, University of Würzburg, Germany) in a 2% antibody with 2% NGS and 0.5% Triton-X 100 in PBS solution. After rinsing the brains five times in PBS for 10 min each, the brains were incubated with the secondary antibody, an anti-mouse antibody from goat with a CF633 dye (Biotium, Hayward, USA) in a 0.4% antibody in PBS with 1% NGS solution, for 2 days at 4°C on a shaker. Afterward, brains were rinsed five times in PBS (10 min each) and dehydrated as described above before clearing in methyl salicylate.

To visualize afferent projections from both the ocelli and the JO, double stained differential tracings were carried out. Anterograde tracings of the antennal afferents were obtained as described above. The lens of the lateral ocelli was removed in the same preparation. A dye droplet (either Dextran tetramethylrhodamine or Dextran, Alexa Fluor 488) was placed inside the ocellar retinae to be taken up by

TABLE 1 Antibody characterization

Antibody	Immunogen	Manufacturer; species; clonality; Cat #; RRID
Synapsin	*Drosophila* Synapsin glutathione-S-transferase fusion protein	E. Buchner, Theodor-Boveri-Institute, University of Würzburg, Germany; mouse; monoclonal; Cat # 3C11 (SYNORF1); RRID: AB_528479

second order afferent neurons. For double staining, combination of Dextran tetramethylrhodamine and Dextran, Alexa Fluor 488 was used. The dyes were incubated for 3–4 h in a dark humid chamber. The subsequent protocol was performed as described above for the pedicel staining.

Finally, all brains were scanned using a confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems GmbH, Wetzlar, Germany) with a 20x water immersion objective (20.0 × 0.7/0.75 NA) obtaining optical sections of 3 or 4 μm thickness. Image stacks were processed using ImageJ 1.52n (Wayne Rasband, National Institutes of Health, USA). To analyze the 3D structure of the JO projections, 3D renders of the tracings were created using the Amira "Voltex" module.

2.4 | Antibody characterization

To locate the central projections of JO and ocellar afferents in neuropils of the *Cataglyphis* brain, a monoclonal antibody to synapsin (SYNORF1, mouse@synapsin; kindly provided by E. Buchner and C. Wegener, University of Würzburg, Germany) was used for visualization of synapse-rich neuropils (Table 1). Synapsin is present in presynaptic terminals and highly conserved among invertebrates. The specificity of the antibody has been characterized previously for *Cataglyphis* ants (Schmitt, Stieb, Wehner, & Rössler, 2016; Schmitt, Vanselow, Schlosser, Wegener, & Rössler, 2017; Stieb, Hellwig, Wehner, & Rössler, 2012; Stieb, Muenz, Wehner, & Rössler, 2010) and most recently for *C. nodus* (Habenstein et al., 2020).

2.5 | Nomenclature

In this study, we refer to Habenstein et al. (2020) (see also https://www.insectbraindb.org for 3D data of the *Cataglyphis* brain) and Ito et al. (2014) for the nomenclature of the neuropils in the ant brain. The nomenclature of the JO and its sensory projections are in line with the nomenclature introduced for the honey bee (Ai et al., 2007).

3 | RESULTS

The antennae of *C. nodus* ants consist of three main segments: the scape is the basal segment located closest to the ant head, the second segment is the pedicel, which contains the JO, and the third is the flagellum, the most distal segment, comprising 10 flagellomeres (Figure 1a). The entire antenna is covered with evenly distributed small bristles. Only on the ventral side of the scape, the density of bristles is very scarce. A hair plate is located close to the joint of scape and pedicel (Figure 1b). At the joint of pedicel and flagellum, evenly distributed dents within the cuticle are located, indicating the location of attachment structures of the JO (Figure 1b).

3.1 | Organization of the JO

3.1.1 | Cross-sections of the pedicel

To get an insight into the anatomy of the JO and its sensory structures inside the antenna of *C. nodus*, the pedicel was sectioned in 1.5 μm thick cross-sections. In total, a pedicel of *C. nodus* produced about 556 cross-sections. The inside of the pedicel contains two antennal nerve branches (anterior (aAN) and posterior antennal nerve (pAN)), two tracheae (anterior (aTR) and posterior trachea (pTR)), and the antennal vessel (AV) that run through the entire antenna (Figure 2). At the distal most end of the pedicel, the JO is attached to the intersegmental membrane of the flagellum-pedicel joint via attachment cells (AC, sometimes called cap cells). These ACs are radially arranged along the cuticle walls of pedicel and flagellum (Figure 2b). The ACs comprise chitin caps (CAP) (Figure 2c). To each cap, one sensory unit of the JO, a so-called scolopidium, is attached (Figure 2d). In each adult worker of *C. nodus*, we investigated (*n* = 4) 40 scolopidia were found. The dendrites of the scolopidia proceed to the cell bodies that attach to the hypodermis of the pedicel in a ring-like manner (Figure 2f-g). In the middle of the pedicel, the axons of the scolopidia converge into three main JO nerves (JON1, JON2, JON3) (Figures 2h-j). At the proximal end of the pedicel, that is, close to the pedicel-scape joint, innervated hairs forming a hair plate (HP, sometimes called Böhm's organ or Böhm's bristles [Böhm, 1911]) are located (Figures 2j-k). Besides the HP bristles, only very rarely innervated bristles were found along the pedicel of *C. nodus*.

3.1.2 | Longitudinal sections of the pedicel

To get a more detailed understanding of the scolopidial structure of the JO in *C. nodus*, the pedicel was sectioned into 1.4 μm thick longitudinal sections. Scolopidia are elongated sensory structures arranged in a circle around the pedicel (see above). A scolopidium consists of an AC with a CAP embedded in the intersegmental membrane (Figures 3a-b). Attached to the caps are chitinous scolopale rods of scolopale cells (Figure 3c). The scolopale cells and rods ensheath the neuronal dendrites of the sensory cells. Each scolopidium comprises three sensory neurons. The total number of sensory neurons of the JO, therefore, was estimated with ∼120. The scolopale dendrites continue to the cell bodies of the three sensory neurons (Figure 3c). At the distal end of the pedicel, no innervated bristles were found (Figure 3).

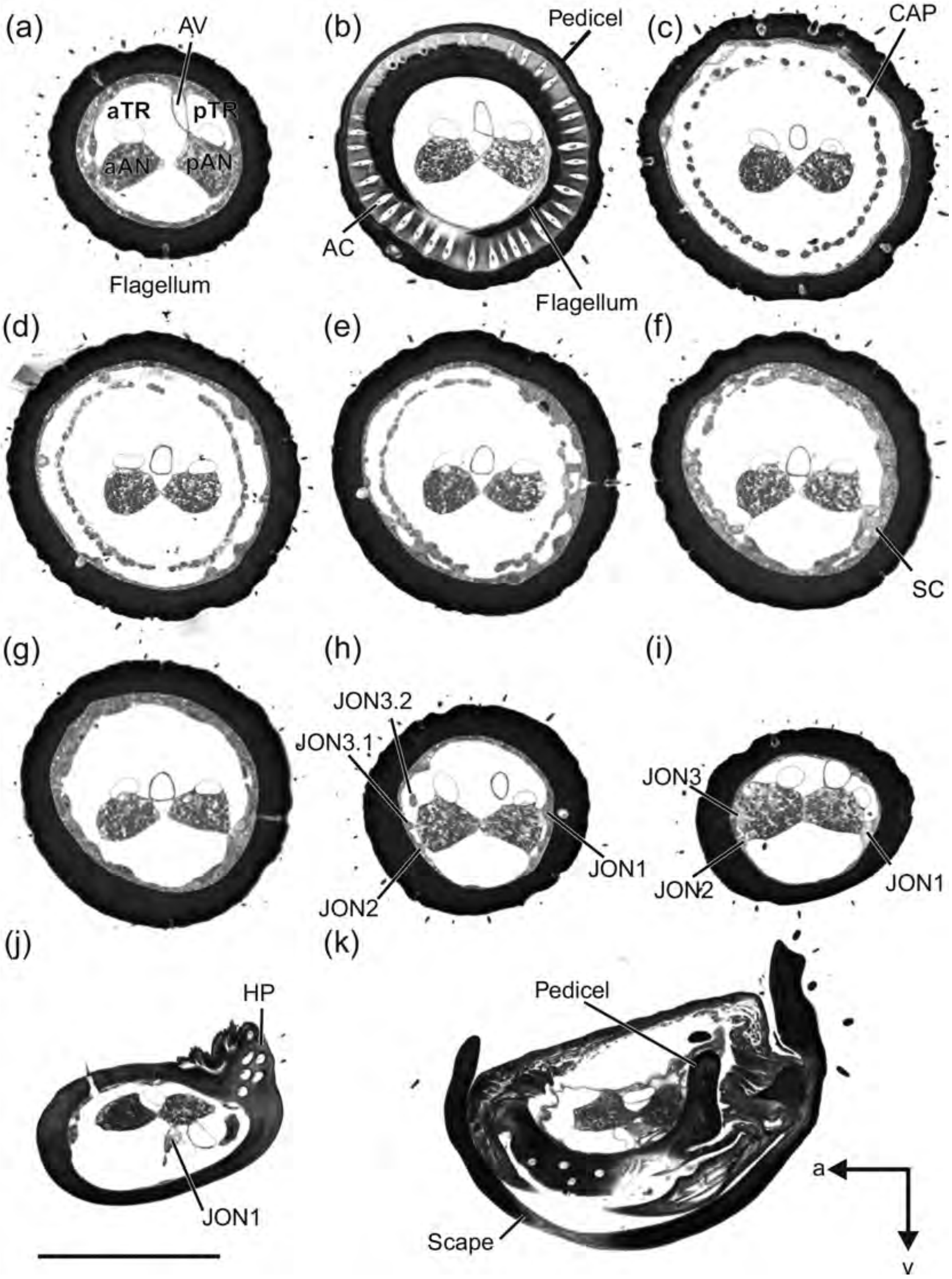

FIGURE 2 Serial cross-sections of the pedicel. The pedicel was sliced into 1.5 μm thick sections. Exemplary sections from the structures within the pedicel are shown. The position of the examples is depicted in the longitudinal views of 3D reconstructions in Figure 4 (bottom). The antennal vessel (AV), two antennal-nerve branches (AN, aAN, anterior antennal nerve; pAN, posterior antennal nerve), and two tracheae (TR, aTR, anterior trachea; pTR, posterior trachea) run along the entire pedicel. (a and b) Starting most distally—in the flagellum—the scolopidia of the Johnston's organ are attached to the intersegmental membrane via attachment cells (AC). (c) Chitin caps (CAP) are located at the most distal end of the scolopidia. (d and e) The elongated mechanosensory units of the JO (scolopidia) are distributed radially within the pedicel. (f and g) The neuronal cell bodies (SC) of the scolopidia are attached to the hypodermis in a circular fashion. (h–j) From there, the afferents of the JO converge into three nerve bundles (JON1-3). (j and k) Close to the joint between pedicel and scape, the hair plate (HP) sensilla are located on the dorsal side of the pedicel. Additional abbreviations: a, anterior; v, ventral. Scale bar 100 μm

3.1.3 | 3D reconstruction of the JO

The axons from sensory neurons within the 40 scolopidia converge in the middle of the pedicel into three JO nerves (JON1, JON2, JON3) (Figures 4 and 5). Each of these three JO nerves innervates a subsection of the JO scolopidia (Figure 4). JON1 innervates 20 posterior scolopidia of the JO and projects along with the pAN (Figures 4 and 5b). The other two nerves run along with and join the aAN. JON2 innervates four ventral JO scolopidia and JON3 16 anterior scolopidia (Figures 4 and 5c-d). The JO nerves project along the two AN branches before joining them close to the joint between pedicel and scape (Figure 5).

3.2 | Central projections of the JO

In *Cataglyphis*, six bundles of axons from the sensory neurons of the antenna project into the brain. Four of these form distinct olfactory sensory tracts (T1-T4) that project into specific subsets of olfactory glomeruli in the antennal lobe (AL) (Stieb, Kelber, Wehner, & Rössler, 2011). Hence, the remaining two sensory tracts containing sensory afferents from the JO will be termed T5 and T6, respectively. This is in line with the antennal sensory tract nomenclature in the honeybee brain (Ai et al., 2007; Maronde, 1991). We used the recently published 3D atlas of the *C. nodus* brain to assign the antennal projections to distinct neuropils in the central brain (Habenstein et al., 2020; for 3D data, see https://www.insectbraindb.org).

As mentioned above, the afferents of the JO project along the two AN branches into the brain. As a first step, we analyzed anterograde tracings of antennal sensory projections that had been mass

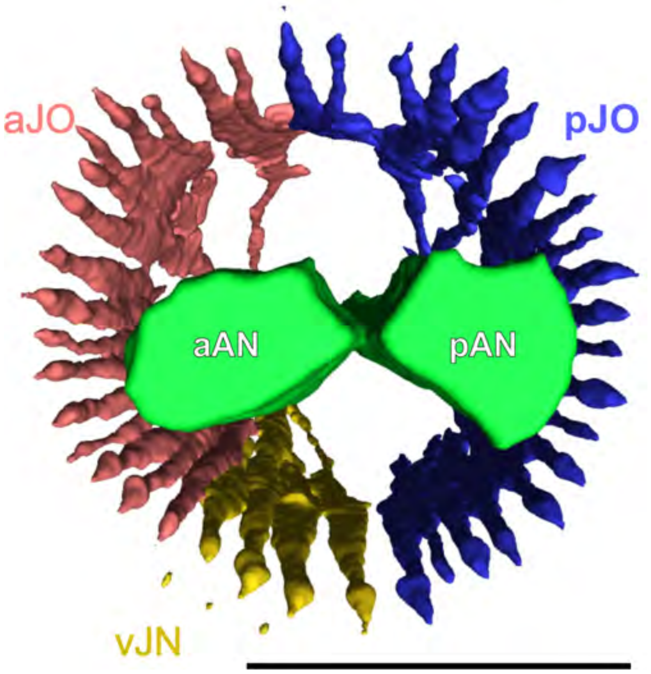

FIGURE 4 3D-reconstruction of the Johnston's organ (view from a distal perspective). The 40 scolopidia of the Johnston's organ (JO) in a *C. nodus* worker antenna (*n* = 4) are radially arranged around the antennal-nerve branches (AN, aAN, anterior antennal nerve; pAN, posterior antennal nerve). The JO scolopidia can be classified into three groups based on their afferent projections (see also Figure 5): The anterior JO (aJO, red) with 16 scolopidia, the posterior JO (pJO, blue) with 20 scolopidia, and the ventral JO (vJO, yellow) with 4 scolopidia. Scale bar 100 μm

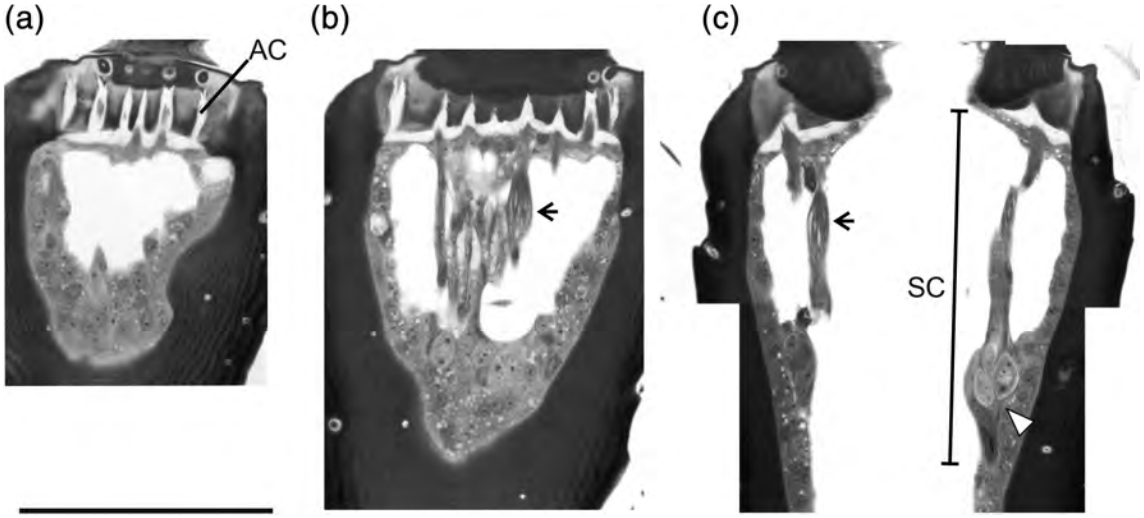

FIGURE 3 Serial longitudinal sections of the pedicel. The pedicel was sliced into 1.4 μm thick sections. (a) The scolopidia of the Johnston's organ are attached to the intersegmental membrane between flagellum and pedicel via attachment cells (AC). (b and c) The scolopidia are elongated mechanosensory structures. Each scolopidium comprises three dendrites from three sensory neurons. Scolopale rods (arrows) surround and enclose the dendrites. Three cell bodies (white arrowhead) of the sensory neurons (scolopale cells (SC)) of an individual scolopidium are attached to the hypodermis. Scale bar 100 μm

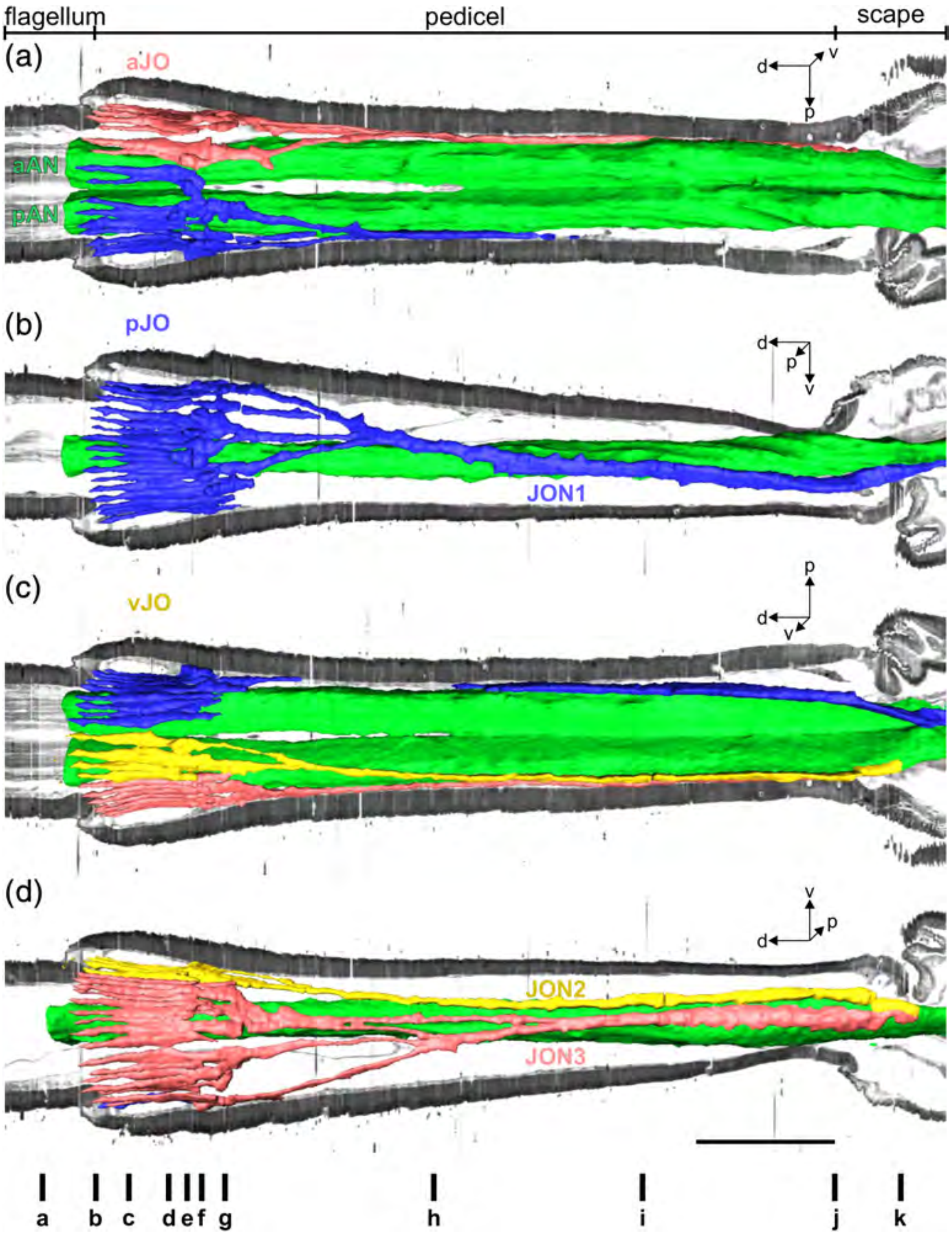

FIGURE 5 3D-reconstruction of the Johnston's organ (longitudinal perspectives). (a) View from dorsal, (b) posterior, (c) ventral, (d) and anterior. The scolopidia of the Johnston's organ (JO) are located in the distal third of the pedicel. Their neuronal afferents converge into three distinct axon bundles. Each axon bundle projects from a specific subset of scolopidia (compare Figure 4). Afferents of the posterior JO (pJO) join into JO nerve 1 (JON1, blue) and run along the posterior antennal-nerve branch (pAN). JO nerve 2 (JON2) contains the afferents of the ventral JO (vJO, yellow) and anterior JO (aJO) afferents join JO nerve 3 (JON3, red). Both JON2 and JON3 run along the anterior antennal-nerve branch (aAN). The positions of the exemplary cross sections shown in Figure 2 are indicated at the bottom (d). Additional abbreviations: d, distal; p, posterior; v, ventral. Scale bar 100 μm

filled by applying fluorescent dye at the level of the pedicel (n = 24). Antennal afferents from the flagellum and pedicel project into several distinct neuropils besides the AL (Figures 6a–e and 7). The respective central neuropils were identified by aligning the anti-synapsin stained neuropils with those described in the *Cataglyphis* brain atlas (Habenstein et al., 2020) (Figure 6f). The majority of antennal mechanosensory afferents projects to the AMMC (Figure 6a). A subset of the projections continues into the saddle (SAD) (Figure 6b-d), and, from there, a proportion of afferents projects into the ventral complex (VX) and the ventrolateral protocerebrum (VLP) (Figure 6b). At the most posterior end, a subset of mechanosensory afferents proceeds further into the posterior slope (PS) (Figure 6d). Another small group of projections goes further down into the gnathal ganglia (GNG) (Figure 6e). 3D reconstructions of the antennal afferents revealed an overview of the two mechanosensory tracts (T5, T6) and their terminal branching fields within distinct brain regions and with respect to other major brain neuropils (Figure 7). The afferents of T5 and 6 bypass the AL to most strongly innervate the AMMC and SAD. From there, a subpopulation of the axons proceeds and terminates in the VX, VLP, and the PS (Figures 6 and 7).

To differentiate the sensory axons associated with receptor neurons of the JO, a double anterograde staining procedure of the AN at the level of the first flagellomere and the pedicel was used (n = 10) (Figure 1c). This differential labeling technique revealed the projection patterns of antennal mechanosensory afferents derived from the JO (labeled in magenta only). The results show that JO afferents contribute to both mechanosensory tracts (T5 and T6, labeled in magenta or red) (Figure 8a). The projections from JO afferents split into two

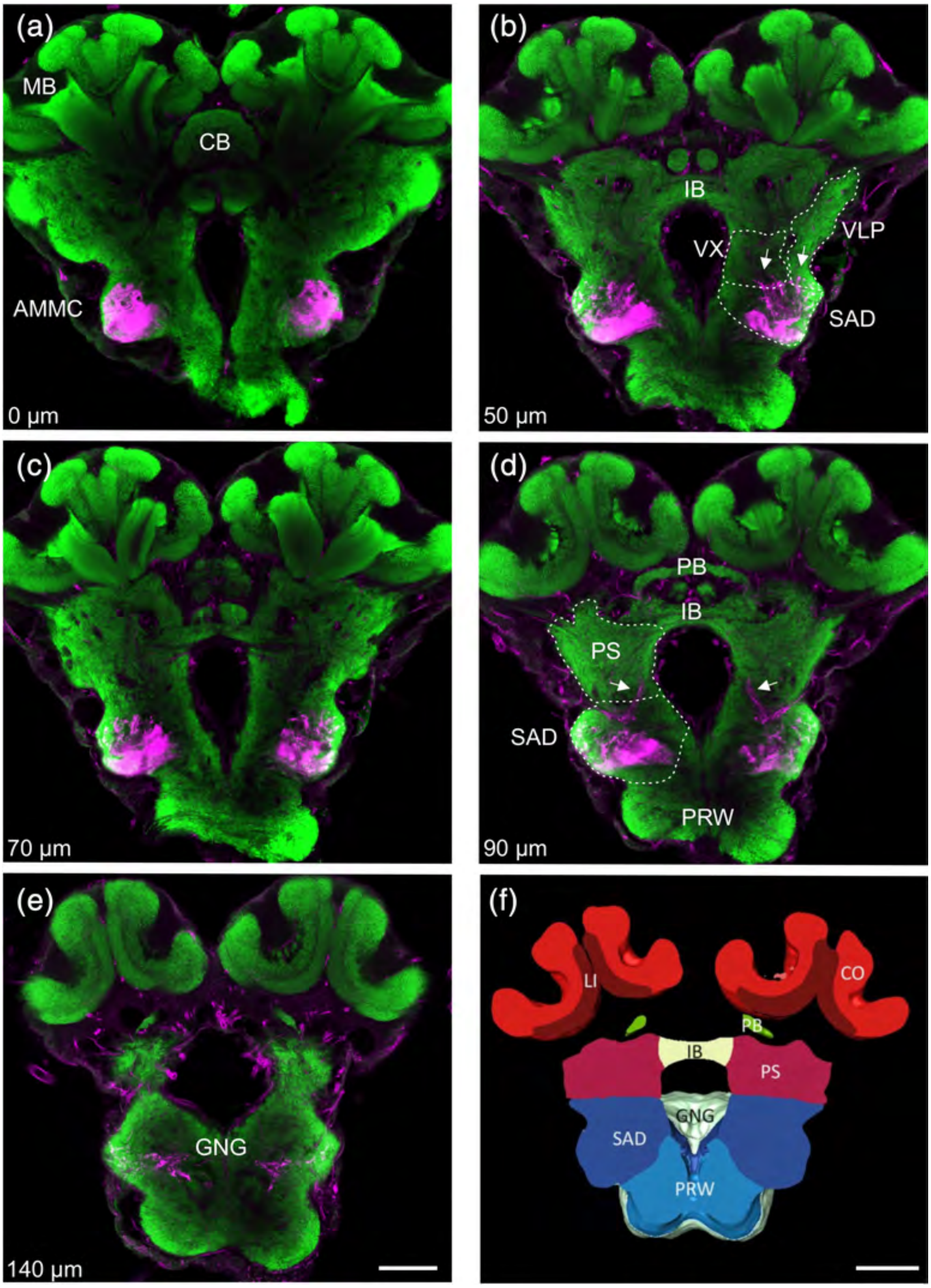

FIGURE 6 Overview of antennal mechanosensory afferent projections in the *C. nodus* brain. For the anterograde staining of antennal afferent projections (magenta), the antenna was cut at the level of the pedicel and a fluorescent dye was applied (see scheme in Figure 1c). The brain was subsequently treated with an anti-synapsin antibody to label synapse-rich neuropils (green). (a–d) The labeled projections (magenta) first converge in the antennal mechanosensory and motor center (AMMC) and, more posteriorly, proceed into the saddle (SAD). The 3D atlas from Habenstein et al. (2020) was used as a reference to identify the central neuropils. (b) Some of the afferents run further to terminate in the ventral complex (VX) and the ventrolateral protocerebrum (VLP). (d and e) At the most posterior end, antennal afferents terminate the posterior slope (PS), and some fibers proceed down into the gnathal ganglia (GNG). (f) Reconstruction of the *C. nodus* brain showing synapsin-rich neuropils and their boundaries at roughly the same depth as in (d) (image from Habenstein et al., 2020). Please note that the section in (d) is slightly tilted compared to the section from the *C. nodus* brain atlas (f), which was taken into account for the identification of synapsin-rich central neuropils. In the lower-left corner of each panel, the depth in relation to (a) is indicated. Each level shown comprises a projection of three optical sections (thickness 3 μm each). Confocal images obtained with 20x objective and 0.75 zoom. Additional abbreviations: CB, central body; CO, collar; IB, inferior bridge; LI, lip; MB, mushroom bodies; PB, protocerebral bridge; PRW, prow. Scale bars in (e) and (f) 100 μm

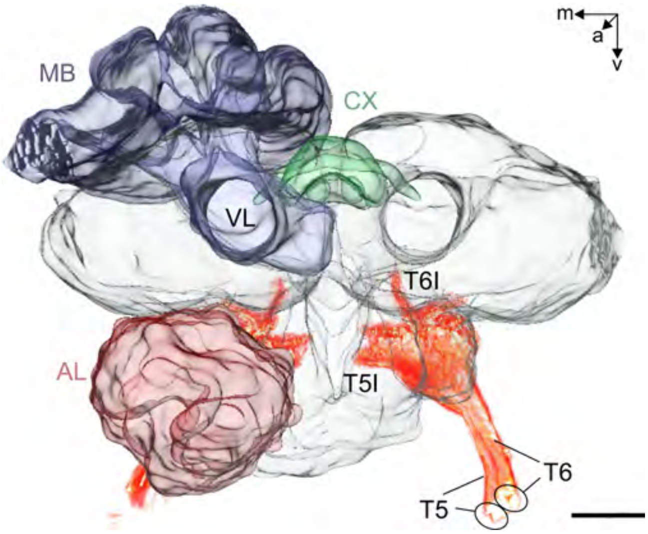

FIGURE 7 3D reconstruction of the projections of antennal mechanosensory afferents in the *C. nodus* brain. The antennal mechanosensory nerve bundles include two fiber bundles from the Johnston's organ (T5, T6). The projections bypass the antennal lobe (AL) and project into the AMMC. From there, a subset of afferents project further into the central brain. T6I projects most posteriorly, while T5I terminates more anteriorly (see more details in Figure 6). Additional abbreviations: a, anterior; CX, central complex; m, medial; MB, mushroom bodies; v, ventral; VL, vertical lobe, . Scale bar 100 μm

terminal branching fields, T5I and T5II at the level of the AMMC (Figure 8b-c). Branches in T5I are ventromedially to the ventral region of the AMMC with some fibers continuing to the SAD, and terminal branches in T5II are dorsolaterally to the dorsal region of the AMMC (Figure 8b). Further axons bypass the AMMC before branching into three collaterals (T6I-T6III). These very likely represent sensory axons exclusively from the JO. The axons forming T6I project most posteriorly and terminate in the PS, whereas T6II and III branches are located in the SAD, VX and VLP (Figure 8c, e-f).

We labeled sensory projections form ocellar neurons to compare their central projections with those from JO afferents. Projections from the lateral ocelli run through the PS and SAD and form branches in the PS, in exactly the same region as terminals from the JO (T6I) (*n* = 16) (Figure 9a). Analyzes of small substacks of optical sections revealed that T6I axons and axons from secondary interneurons of the ocelli are in very close apposition within the same region in the PS (*n* = 6) (Figure 9b). Some of the overlapping branches may proceed further into the SAD. Axons of T6II terminate in the VX, whereas T6III axons terminate in the VLP region (Figure 8b).

3.3 | Organization of the JO in virgin queens and males

As a first step to compare the JO of the flight enabled reproductive castes with the ambulatory worker caste of *C. nodus*, we produced exemplary serial cross-sections of the pedicel of virgin queens and males. To estimate potential differences of the antennal segments, scanning EM images were obtained. These show that the pedicel of worker ants is 157 μm in diameter and 627 μm in length (*n* = 1) (Figure 10a-c). In queens, the pedicel is 169 μm in diameter and 639 μm in length (*n* = 1) (Figure 10d-f). The pedicel of males is the largest with 195 μm in diameter and 652 μm in length (*n* = 1) (Figure 10g-i).

As in *C. nodus* workers, the JOs of the reproductive castes are arranged radially within the distal portion of the pedicel. Here, at the joint between pedicel and flagellum, the scolopidia of the JO are attached to the intersegmental membrane. In the example of a *C. nodus* male we found 48 (*n* = 1) scolopidia, while the virgin queen had 42 (*n* = 1) (Figure 10). Both, males and queens, have three sensory neurons associated with each scolopidium. Based on this, the number of sensory neurons in the JO can roughly be estimated with ~144 in males and ~ 126 in queens. In workers and queens, the diameter of the ANs is similar, while the ANs in males are strikingly thicker. Overall, the pedicel of males is more densely packed with cellular tissue than their female counterparts (Figure 10).

4 | DISCUSSION

This study, for the first time, analyzes in detail the three dimensional structure of the JO in the antenna of an ant of the genus *Cataglyphis* (desert ants) together with its afferent projections into the central brain of the ant. The JO scolopidia are attached to the intersegmental membrane between flagellum and pedicel via the chitinous caps of the attachment cells. Scolopale rods that are attached to the caps ensheath three neuronal dendrites in each scolopidium. In *Cataglyphis* workers, the 40 scolopidia are arranged radially within the pedicel. They can be classified into three groups based on their afferent innervations. JO sensory neurons project into the AMMC and the SAD via the T5 fiber bundle. The T6 projections from the JO bypass the AMMC, proceed via the SAD, and terminate in the VX, VLP, PS, and GNG. Projections from the ocelli have branches in close apposition with JO terminals within the PS. While the overall structure of the JO is similar between the different castes of *Cataglyphis*, the number of scolopidia differs mainly between males and the two female castes.

4.1 | Morphology of the JO

The JO of *C. nodus* is located at the distal end of the second antennal segment, the pedicel. It is attached to the intersegmental membrane at the joint of pedicel and flagellum. The attachment sites of its subunits can be seen on the pedicel cuticle as small pits. This general morphology of the sensory units in the JO of *C. nodus* resembles the JOs of most other insect species investigated (review: Yack, 2004). In *C. nodus*, each JO scolopidium contains three sensory neurons. This is in line with most other insects, with the exception of only a few Diptera with only two sensory neurons per scolopidium (Schmidt, 1974;

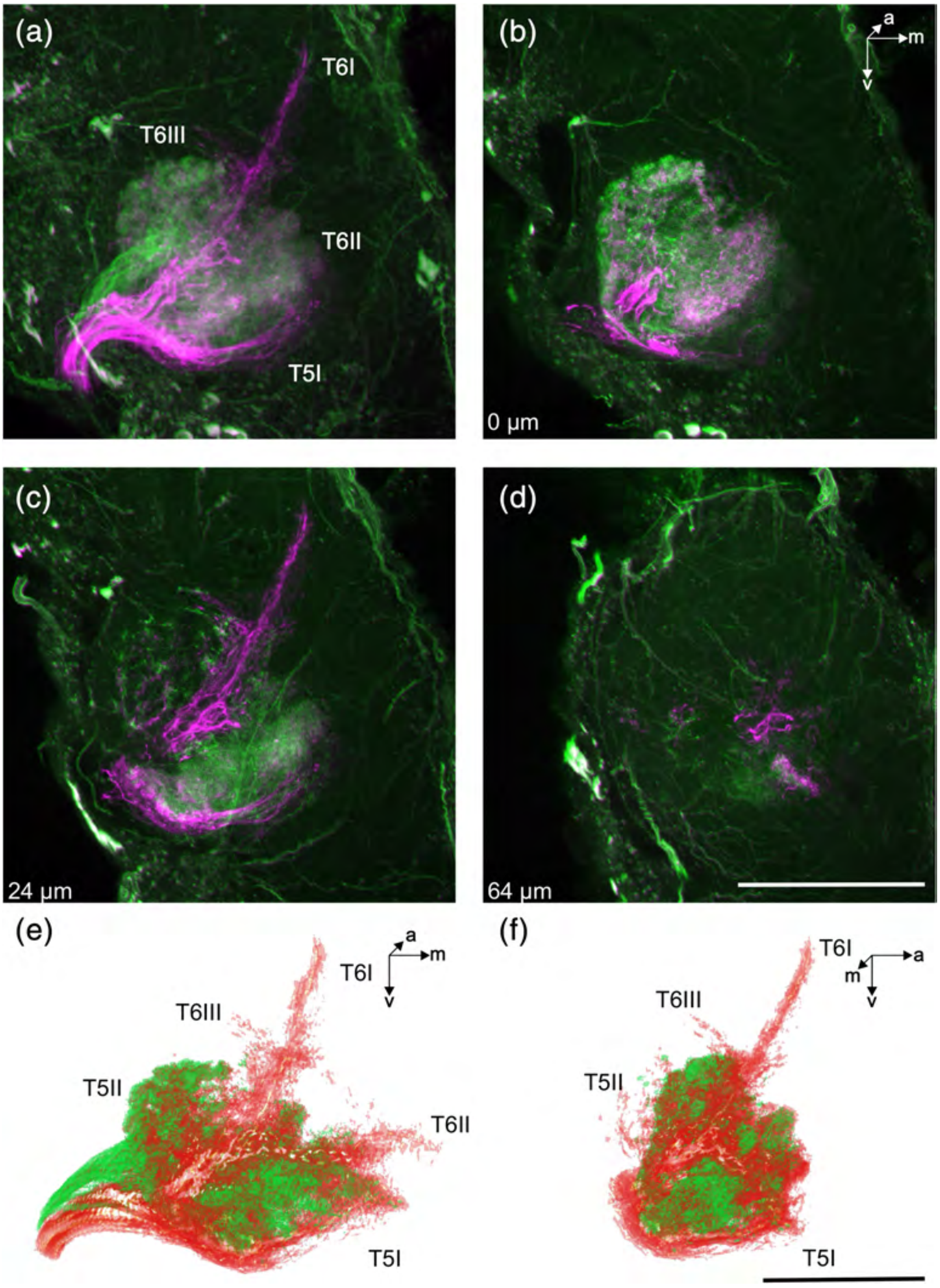

FIGURE 8 Differential tracing of Johnston's organ afferents and 3D-reconstruction. The Johnston's organ (JO) afferents were selectively stained using an anterograde double-tracing procedure (see methods and Figure 1c). Mechanosensory projections of the antenna are shown in green or merged green/magenta, while projections of the JO are labeled in magenta only. (a) Overview of the differentially labeled projections of the JO (magenta). While most other antennal mechanosensory projections terminate in the antennal mechanosensory and motor center (AMMC) (b) and saddle (SAD) (c), the two fiber bundles of the JO (within T5 and T6) proceed further into more posterior brain areas. T5 splits into two branches with T5I projecting to the ventral region of the AMMC (b) with some fibers continuing to the SAD (c), and T5II projects to the dorsal region of the AMMC (b). T6 bypasses the AMMC and splits into three branches. T6I terminates most posteriorly in the PS (c) (compare with Figure 6). T6II terminates in the ventral complex (VX) and T6III in the ventrolateral protocerebrum (VLP) (c). Some neurites project further ventrally into the gnathal ganglion (GNG) (d). (b–d) In the lower left corner of each panel, the depth relative to (b) is indicated. Each panel shows a projection of optical sections (thickness of 3 μm each) (a) comprises a projection of 83 optical sections, (b, c) comprise a projection of four optical sections, respectively; (d) comprises a projection of six optical sections). Confocal images using a 20x objective and 2.40 zoom. (e, f) Two views of 3D-reconstruction of the JO afferents (red) and other antennal mechanosensory projections (green). Scale bars in (d) and (f) 100 μm

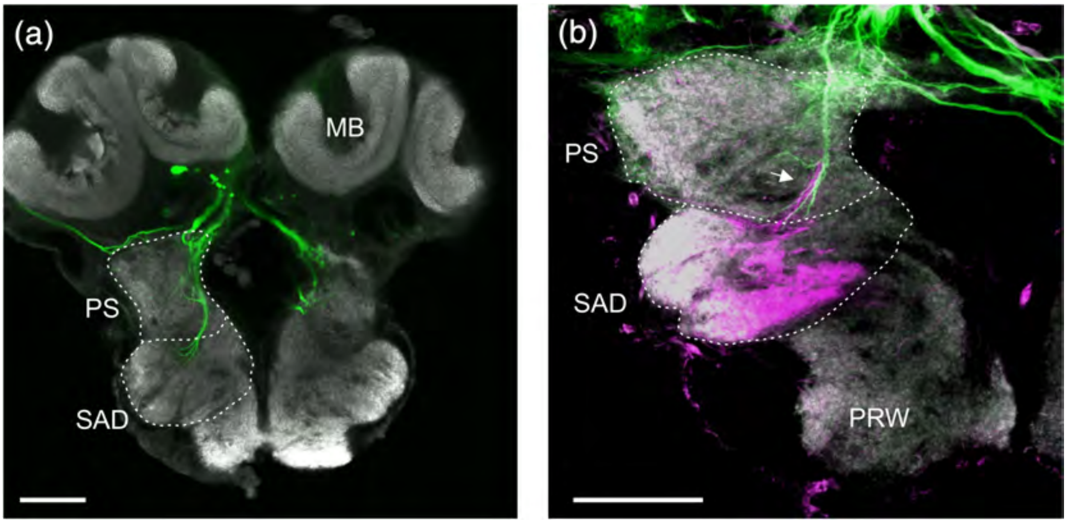

FIGURE 9 Differential tracing of afferents from the lateral ocelli and the Johnston's organ. (a) Afferent neurons from the lateral ocelli (green) project into the posterior slope (PS) and the saddle (SAD). (b) In the PS, projections from the ocelli (green) and Johnston's organ projections terminate in very close proximity to each other. Confocal images using a 20x objective and (a) a 0.75 zoom or (b) a 1.50 zoom. Each panel shows a projection of three optical sections (thickness of 3 μm each). Additional abbreviations: MB, mushroom bodies, PRW, prow. Scale bars in (a) and (b) 100 μm

Uga & Kuwabara, 1965). With a total number of 40 scolopidia, the number of scolopidia in the JO of adult *C. nodus* workers is rather low, compared to other insects. In general, the JO exhibits a wide range in scolopidial numbers and associated sensory neurons across the insects (Snodgrass, 1926). While the JO of *C. nodus* contains only 120 sensory neurons, the JO of mosquitos may hold up to 30,000 sensory neurons (Boo & Richards, 1975). Even when compared to smaller JOs, like the one in *D. melanogaster* with 720 sensory neurons, the numbers in the JO of *Cataglyphis* are still rather small. Interestingly, also other Hymenoptera were shown to possess JOs comprising small cell numbers (Snodgrass, 1926; Vowles, 1954). Honeybees are in a comparable range like *D. melanogaster* with about 720 neuronal cells in 240 scolopidia (Ai et al., 2007), and the JO of sawflies consists of about 750 neuronal cells in 250 scolopidia (Hallberg, 1981). In contrast to ant workers, however, mosquitos, fruit flies, bees, and sawflies are capable of flight. The JO is an important organ for flight control and helps flying insects to keep their posture during flight (Sane et al., 2007). Surprisingly, however, the sexual castes of *C. nodus* that are capable of flight (male and queen), only show a slightly higher number of scolopidia and associated sensory neurons in the JO. Therefore, a stronger effect on the number of scolopidia in the JO may be ascribed to the capability of hearing. While mosquitos (Cator et al., 2009; Göpfert & Robert, 2001a), fruit flies (Göpfert & Robert, 2001b; Göpfert & Robert, 2002), and honeybees (Dreller & Kirchner, 1993a; Dreller & Kirchner, 1993b; Dreller & Kirchner, 1995) detect airborne sound using the JO, ants are deaf (Roces & Tautz, 2001) suggesting that the JO of ants does not serve as a sensory organ for the detection of airborne sound. Compared with other ant species investigated so far, the dimensions of the JO in *Cataglyphis* are much more similar. *Formica* wood ants have a JO comprising only 20 scolopidia (about 60 sensory neurons) (Vowles, 1954), and *Camponotus* (carpenter) ants possess about 55 scolopidia with 165 estimated sensory neurons (Masson & Gabouriaut, 1973).

Similar to all species studied so far, the scolopidia in *C. nodus* are attached to the intersegmental membrane by chitin caps, which are connected to scolopale rods. Antennal deflection is transferred via these structures and, as a result lead to the opening of mechanosensitive ion channels in the sensory dendrites upon stimulation. The resulting action potentials are relayed to the central brain (Todi et al., 2004). In *C. nodus*, the afferent projections of sensory neurons within scolopidia of the JO are bundled into three distinct nerves. Similar to the situation in *A. mellifera* (Ai et al., 2007), these three JO nerves are supplied by specific subsets of scolopidia, respectively. The posterior scolopidia of the JO in bees (Ai et al., 2007) and *C. nodus* (approx. half of the total number of scolopidia) join JON1, the anterior scolopidia converge into JON2, and ventral scolopidia into JON3. However, whereas the scolopidial subgroups are clustered within the pedicel of *A. mellifera* (Ai et al., 2007), in *Cataglyphis* they are very neatly aligned along an evenly spaced circle within the pedicel. This radial arrangement of the scolopidia can also be found in other insects, including other ant species (Masson & Gabouriaut, 1973; Vowles, 1954), fruit flies (Schmidt, 1974), and sawflies (Hallberg, 1981).

4.2 | Neuronal projections of the JO

The overall neuronal projection patterns of the JO afferents in *C. nodus* show a high degree of similarity with those described in the honeybee (Ai et al., 2007; Brockmann & Robinson, 2007) and *Drosophila* (Kamikouchi et al., 2006). The most prominent part of the JO afferents projects to the AMMC (T5 tract). There the JO afferents

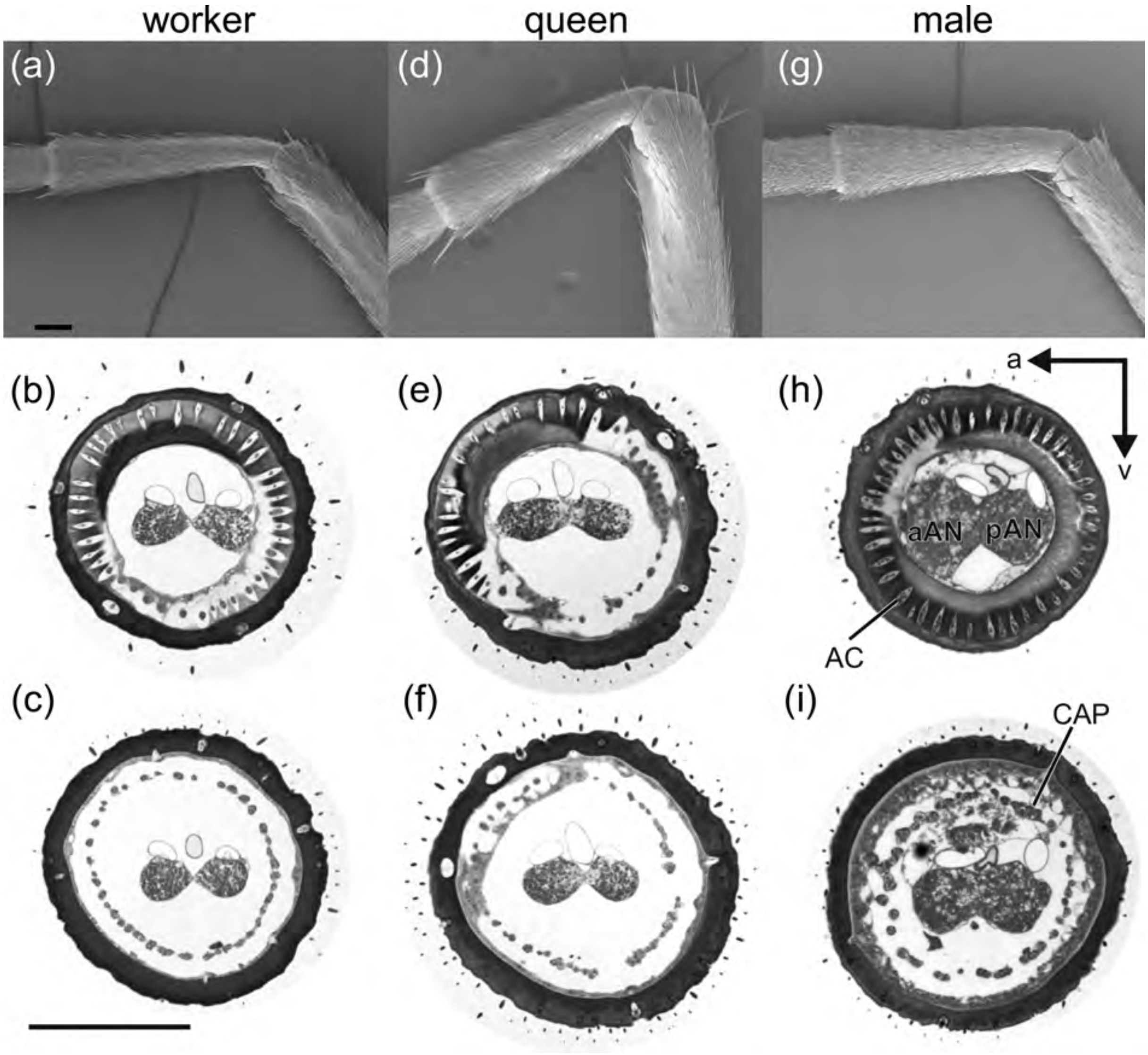

FIGURE 10 Comparison of the pedicel and the Johnston's organ between a *C. nodus* worker, queen and male. Polymorphism between the two female castes and males in *C. nodus*. (a–c) Worker, (d–f) queen, and (g–i) male. (a, d, g) Scanning electron microscopy images of the pedicel in the two females castes and males. The pedicel of workers (a) is smaller compared to the situation in queens (d). The longest and widest pedicel was found in males (g). (b, c, e, f, h, i) Exemplary cross-sections at different levels of the pedicel (1.5 μm thickness). While the overall structure of the Johnston's organ (JO) is similar in the female castes and males, the number of scolopidia differs slightly. (b and c) The JO in the worker antenna comprises 40 scolopidia (*n* = 4), (e and f) the JO of a queen comprises 42 scolopidia (*n* = 1), and (h and i) the JO of a male comprises 48 scolopidia in the JO (*n* = 1). For details see text. Abbreviations: aAN, anterior antennal nerve; AC, attachment cells; CAP, scolopale cap; pAN, posterior antennal nerve. (a–c) For better comparison, the exemplary images of the worker antenna were taken from Figures 1 and 2. Scale bars in (a) and (c) 100 μm

form a somatotopic map in *Drosophila* (Kamikouchi et al., 2006), honey bees (Ai et al., 2007), and mosquitos (Ignell, Dekker, Ghaninia, & Hansson, 2005). In *Drosophila*, antennal deflection by wind is represented in a map-like manner in the AMMC (Yorozu et al., 2009). Wind compass information is then transferred to the central complex (CX) (Okubo et al., 2020). In the CX, wind information is integrated into the path integrator of the insect brain (Honkanen, Adden, Freitas, & Heinze, 2019), where it is combined with visual cues to allow for multimodal spatial orientation (Okubo et al., 2020).

The T6 nerve tract of the JO afferents bypasses the AMMC and projects into more posterior neuropils. In *C. nodus*, JO afferents terminate in the VX (T6II) and the VLP (T6III). These projections show similarities with those in *Drosophila* (Kamikouchi et al., 2006) and the honeybee (Ai et al., 2007). Selective staining of individual JO nerves in the honeybee revealed that subgroups of JO scolopidia (pJO, aJO, vJO) have similar overall projection patterns, and only terminal branches of vJO in T6I were segregated from those of aJO and pJO (Ai et al., 2007). Whether this is also the case in *Cataglyphis* could not be resolved with our double staining technique. The pedicel of *Cataglyphis'* antennae is very thin and surrounded by thick cuticle, which renders access to individual JO nerves or sensory neurons impossible. It therefore remains unclear whether the three groups of

JO receptor neurons represent functional groups or, alternatively, the projections via three nerves may reflect a developmental pattern. Ideally, future studies might be able to combine functional analyzes with projections of individual JO receptor neurons using intracellular recording and staining from a more proximal position of the nerves to resolve this.

In *Cataglyphis*, both the VX and VLP receive visual input from the primary optic ganglia (medulla, lobula) via the posterior (POC) and inferior (IOC) optic commissures (Habenstein et al., 2020). In the honeybee, similar visual projections into this area were found (Maronde, 1991). Interestingly, in the PS terminals of the T6I tract from the JO converge with terminals of sensory neurons from the ocelli. A similar finding has been reported in the honeybee (Ai et al., 2007; Pareto, 1972). In *Cataglyphis*, the ocelli were shown to be polarization sensitive (Fent & Wehner, 1985; Mote & Wehner, 1980) and the photoreceptor neurons contain untwisted rhabdomeres (Penmetcha, Ogawa, Ribi, & Narendra, 2019). This indicates that multimodal navigational cues from the ocelli and the JO converge in this brain region. Whether the two sets of neurons directly synapse on each other or, more likely, converge on multimodal interneurons still needs to be shown using EM techniques. Due to their multisensory input from different primary (visual and mechanosensory) channels, the PS was recently defined as another multisensory integration center in the *Drosophila* brain (Currier & Nagel, 2020). Our results indicate that in addition to the PS, the VX and VLP may also serve as multimodal integration regions.

4.3 | JO as a multisensory organ in *Cataglyphis*

4.3.1 | Flight control or sexual dimorphism?

Since the JO of ants contains a rather small number of scolopidial units and is not involved in hearing, it is a perfect candidate to study its potential involvement in other sensory modalities. Additionally, ants provide the unique feature to compare the JO in ambulatory and flying individuals within the same species. The reproductive castes of *Cataglyphis* ants are winged and capable of flight, while the worker caste is purely ambulatory (Peeters & Aron, 2017). Interestingly, our first results show that the difference in the number of JO scolopidia between ambulatory and flying *C. nodus* is only marginal. Workers have the lowest (and very invariant) number of scolopidia, followed by an only marginally higher number in a queen, but a clearly higher number in a male. This result corresponds with findings in honeybees (McIndoo, 1922). *A. mellifera* workers have, as in *C. nodus*, the lowest number of scolopidia with about 70 scolopidia (in *C. nodus* 40 scolopidia). This is closely followed by honeybee queens with 72 (in *C. nodus* 42) and with some distance the drones (males) with 100 caps in the intersegmental membrane (in *C. nodus* 48 scolopidia) (McIndoo, 1922). Importantly, in the honeybee, both female castes and males are capable of flight. However, in both the honeybee and in *C. nodus*, only the number of scolopidia in males is substantially higher compared to the situation in both female castes. This suggests that in both cases, the honeybee and the ant, the differences in scolopidia numbers are not linked to the capability of flight. However, the JO could still play a role during *Cataglyphis'* courtship, which requires further investigation.

4.3.2 | JO as a wind compass

Elaborated navigational skills play a crucial role in the survival of foraging ants. In addition to their largely visually guided path integrator, *Cataglyphis* ants also employ orientation strategies based on information about wind direction. This allows the ants to find the scattered food items in their meager environment, to pinpoint their nest entrance (Steck et al., 2009; Wolf & Wehner, 2000), and to navigate at night (Wehner & Duelli, 1971). The ants are even able to track their displacement by wind and correct for it (Wystrach & Schwarz, 2013). Especially in their desert and desert-like habitats, these displacements can be quite significant. Desert ants constantly track wind directions in order to account for dislocations (Wystrach & Schwarz, 2013). Previously, it was shown by manipulation experiments that the antennae and especially the ability to move the joints of the antennal segments are essential for anemotactic orientation in desert ants (Wehner & Duelli, 1971; Wolf & Wehner, 2000). This makes the JO the most likely candidate for a wind compass in *Cataglyphis* ants.

The JO was also shown to detect multiple modalities and can be viewed as a multisensory organ. In *D. melanogaster*, the JO is able to distinguish between sound and wind information (Yorozu et al., 2009). The different stimuli activate different subsets of sensory neurons within the JO. Sound-sensitive neurons in the JO are phasically activated, while wind-sensitive neurons are tonically activated (Yorozu et al., 2009). Interestingly, each scolopidium in *D. melanogaster* houses one sound- and one wind-sensitive neuron (Ishikawa, Fujiwara, Wong, Ura, & Kamikouchi, 2020). The different sensory neurons also project to distinct parts of the AMMC, and wind direction is even represented in a map-like manner (Yorozu et al., 2009). These properties allow the JO to function as a multisensory organ. Wind compass information in *Drosophila* is conveyed from the terminal regions of the T6 tract into the CX (Okubo et al., 2020), a center for path integration in the insect brain (Honkanen et al., 2019). Interestingly, our results show that JO afferents in *Cataglyphis* contribute to two distinct projection regions within the AMMC. Future studies will be necessary to determine, if the JO afferents in *C. nodus* terminate in a map-like manner in the AMMC as they do in *Drosophila* (e.g. Yorozu et al., 2009).

4.3.3 | JO as gravity detector

While *D. melanogaster* is typically described as having two sensory neurons in each scolopidium of the JO (Uga & Kuwabara, 1965), more recent studies have shown that a significant subset of scolopidia comprise three neuronal cells (Todi et al., 2004). Todi et al. (2004) proposed that these additional sensory neurons could be used for sensing the gravitational force. Also in ants, the JO has been proposed to be involved in graviception (Beckingham, Texada, Baker, Munjaal, & Armstrong, 2005; Vowles, 1954). For desert ants, the reception of the gravitational force plays an important role during path integration. *Cataglyphis* measures the walked distance during foraging trips via a step integrator (Wittlinger, Wehner, & Wolf, 2006). However, to estimate the correct distance from the nest, *Cataglyphis* has to take the slopes on its way into account and integrate this information into its path integrator (Grah & Ronacher, 2008; Grah, Wehner, & Ronacher, 2005; Ronacher, 2020). In contrast to other ants (Markl, 1962; Markl, 1963), bristle fields on the ants' body do not seem to be involved in the reception of gravitational forces in *Cataglyphis* (Wittlinger, Wolf, & Wehner, 2007). Up to now, the mechanism by which *Cataglyphis* measures slopes still remains elusive (Ronacher, 2020). The JO is a promising candidate to fulfill this function in gravity reception. In fact, in *D. melanogaster* the primary sensory organ for detecting gravity seems to be the JO (Kamikouchi et al., 2009). The ring-like arrangement of the scolopidia in the JO in *Cataglyphis* might even promote this function. Due to the different location of the scolopidia, some of the sensory neurons would always be maximally stretched, independent of antennal movement (Kamikouchi et al., 2006). This would allow the JO to distinguish between several tonic sensory inputs. It is likely, however, that other mechanosensory receptors, especially on the legs, like hair plates at the leg joints, or stretch receptors inside the leg, could be involved in gravity perception. Future studies are needed to investigate the involvement of the JO and other mechanoreceptors in the slope estimation in *Cataglyphis* ants.

4.3.4 | Potential multimodal contributions of the JO to navigation in *Cataglyphis*

The JO is a highly multimodal sensory organ involved in flight control and the reception of sound, wind, and gravitation. Its afferent projections in *Cataglyphis* extend beyond the AMMC into other parts of the central brain including the SAD, VX, VLP and PS. Interestingly, the PS was recently considered as a multimodal integration center in *Drosophila* (Currier & Nagel, 2020). It receives input from the ocelli in *Cataglyphis* and also in the honeybee (Ai et al., 2007; Pareto, 1972). Since the ocelli in *Cataglyphis* are sensitive to polarized light (Penmetcha et al., 2019), the convergences of information from the JO (possibly a wind compass and a graviceptor) with information from the sun compass would allow the PS to be a suitable candidate neuropil to synchronize or even calibrate these two compass systems. Additionally, the terminal areas of T6 afferents in the VX and VLP may also converge with information from afferent projections from the optic lobes (Habenstein et al., 2020; Ibbotson & Goodman, 1990; Maronde, 1991). This might allow for processing both input from the visual surrounding (panorama) and directional (polarized) skylight cues with information from the JO. The combination of visual information, sky compass information, as well as wind compass information from the JO, might allow naïve *Cataglyphis* ants to calibrate their visual navigational systems with geostable directional reference systems such as gravitational forces. This calibration is crucial for naïve ants to become successful navigators (Fleischmann, Christian, Müller, Rössler, & Wehner, 2016; Fleischmann, Grob, Wehner, & Rössler, 2017; Fleischmann, Rössler, & Wehner, 2018; Wehner, Meier, & Zollikofer, 2004). During the first excursions outside of the nest, the ants have to calibrate their compass systems and learn their surroundings (Grob, Fleischmann, & Rössler, 2019). These learning walks also correlate with structural synaptic plasticity along two visual pathways (Grob, Fleischmann, Grübel, Wehner, & Rössler, 2017; Rössler, 2019). Interestingly, in contrast to experienced foragers, *C. nodus* does not use celestial compass cues during first learning walks outside the nest entrance (Grob et al., 2017)—at the transition from inside the dark nest to outdoor foraging. During this phase the ants rather rely on the earth's magnetic field as their sole compass cue (Fleischmann, Grob, et al., 2018). This suggests that the ants use the earth's magnetic field as an earthbound reference to calibrate their internal celestial compass (review: Grob et al., 2019). The multimodal nature of the JO makes it a suitable candidate to play a crucial role during learning walks. In addition, the insect antennae have been suggested as a potential site for magnetoreception (de Oliveira et al., 2010; Guerra, Gegear, & Reppert, 2014; Lucano, Cernicchiaro, Wajnberg, & Esquivel, 2006), which renders the antenna and potentially the JO as one candidate in the search for the insect magnetic compass (Fleischmann, Grob, & Rössler, 2020).

Future combinations of behavioral manipulations with physiological and anatomical studies in *Cataglyphis* ants are highly promising to further elucidate the roles of this fascinating multisensory organ in navigation and the respective processing areas in the central brain of the ant.

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AUTHOR CONTRIBUTIONS

Robin Grob, Pauline N. Fleischmann, and Wolfgang Rössler conceived the study. Robin Grob, Claudia Groh, Christian Stigloher, and Wolfgang Rössler designed the anatomical analyzes of the JO. Robin Grob, Kornelia Grübel, Pauline N. Fleischmann, and Wolfgang Rössler designed the anterograde tracing section of the study. Robin Grob, Clara Tritscher, and Kornelia Grübel performed the experiments, collected the data and, together with Wolfgang Rössler and Pauline N. Fleischmann, analyzed and discussed the data. Wolfgang Rössler was responsible for funding acquisition for this study. Robin Grob drafted and Wolfgang Rössler and Pauline N. Fleischmann revised the manuscript. All authors approved the final version of the manuscript for submission.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Raw confocal, histology, scanning-electron microscopy images and 3D data are available from the authors upon reasonable request. For comparison of brain data, 3D data of the brain of Cataglyphis nodus (from Habenstein et al., 2020) are available at the Insect Brain Database website (https://www.insectbraindb.org/).

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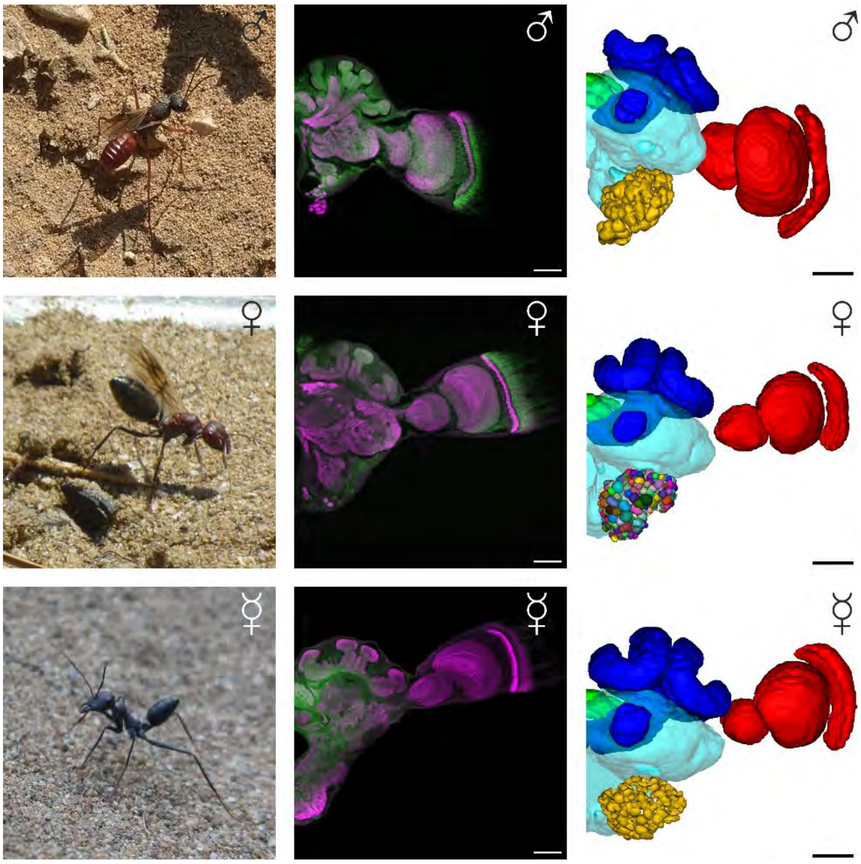
5 Manuscript 4: Sex-Specific and Caste-Specific Brain Adaptations Related to Spatial Orientation in *Cataglyphis* Ants



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RESEARCH ARTICLE

Sex-specific and caste-specific brain adaptations related to spatial orientation in *Cataglyphis* ants

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Abstract

Cataglyphis desert ants are charismatic central place foragers. After long-ranging foraging trips, individual workers navigate back to their nest relying mostly on visual cues. The reproductive caste faces other orientation challenges, i.e. mate finding and colony foundation. Here we compare brain structures involved in spatial orientation of *Cataglyphis nodus* males, gynes, and foragers by quantifying relative neuropil volumes associated with two visual pathways, and numbers and volumes of antennal lobe (AL) olfactory glomeruli. Furthermore, we determined absolute numbers of synaptic complexes in visual and olfactory regions of the mushroom bodies (MB) and a major relay station of the sky-compass pathway to the central complex (CX). Both female castes possess enlarged brain centers for sensory integration, learning, and memory, reflected in voluminous MBs containing about twice the numbers of synaptic complexes compared with males. Overall, male brains are smaller compared with both female castes, but the relative volumes of the optic lobes and CX are enlarged indicating the importance of visual guidance during innate behaviors. Male ALs contain greatly enlarged glomeruli, presumably involved in sex-pheromone detection. Adaptations at both the neuropil and synaptic levels clearly reflect differences in sex-specific and caste-specific demands for sensory processing and behavioral plasticity underlying spatial orientation.

KEYWORDS
antennal lobe, central complex, learning and memory, mushroom bodies, optic lobes, polymorphism, synaptic plasticity

1 | INTRODUCTION

Cataglyphis desert ants are well adapted to the harsh environment of arid habitats where ground temperatures regularly reach up to 70°C. Unlike many other desert dwellers, *Cataglyphis* workers do not avoid the burning heat, but rather forage during the hottest hours of the day. To avoid staying out in the sun for too long, the ants show impressive navigational capabilities (Wehner, 2020). For that reason, *Cataglyphis* foragers have been well-studied experimental models for the neuroethology of spatial orientation and navigation for several decades (Wehner, 2019). Desert ants use a wide range of navigational strategies to not get lost during their far-ranging foraging runs (Wehner, 2020). Their navigational repertoire includes path integration based on a step integrator for estimating distance (Wittlinger et al., 2006) combined with a celestial (Müller & Wehner, 1988) or magnetic (Fleischmann, Grob, et al., 2018) compass for determining

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direction. In addition, desert ants use visual landmarks (Buehlmann et al., 2012; Fleischmann et al., 2016; Steck et al., 2009), panoramic scenes (Fleischmann, Rössler, & Wehner, 2018) and learned routes (Collett, 2010) for finding their ways back home. Before the ants become foragers, they go through an age-related sequence of different behavioral stages (age-related polyethism). Female *Cataglyphis* workers start out as more or less motionless callows, then take care of the brood, act as food storage units, bring out waste and help to maintain the nest (Schmid-Hempel & Schmid-Hempel, 1984). After about 4 weeks of indoor tasks, workers leave the nest to calibrate their visual guidance systems (Grob et al., 2019). For that, they perform well-structured learning walks around their nest entrance while using the earth's magnetic field as a reference system (Fleischmann et al., 2020b; Zeil & Fleischmann, 2019). After up to 3 days of learning walks, the ants go out and forage for their colony (Schmid-Hempel & Schmid-Hempel, 1984). Consequently, workers have to be equipped with plastic brains that flexibly support visual-learning based behavioral tasks (Grob et al., 2017; Habenstein et al., 2020, 2021; Schmitt et al., 2016; Stieb et al., 2012).

While the worker caste only includes female ants, the reproductive caste comprises both sexes (Wilson & Hölldobler, 2005). In the genus *Cataglyphis*, the social structure and the ways of division of reproduction are diverse. *Cataglyphis* colonies may have single or multiple reproductive queens, which can be either singly or multiply mated. In other cases, workers may also be able to produce offspring (Boulay et al., 2017). *Cataglyphis* ants perform different mating (Leniaud et al., 2011) and dispersal strategies (Peeters & Aron, 2017). This diversity during mating and dispersal allowed *Cataglyphis* to populate a vast distributional range of arid habitats with many different species (Wehner, 2020). In any case, reproductive and worker castes face vastly different behavioral tasks characterized by specific orientation challenges. In contrast to some *Cataglyphis* species, both sexes of the reproductive caste in *C. nodus* are winged and fly out from their nest of origin. This could indicate formations of "mating swarm" (also called "male aggregation") mating behavior, where reproductive animals of several colonies meet at some distance from their natal nest to mate (Peeters & Aron, 2017). Afterward, mated queens found a new colony on their own. In contrast, workers are purely ambulatory and undergo an age-related polyethism from performing interior tasks to outdoor foraging. When males fly out of their natal nest, their primary goal is to find a virgin queen (gyne) and mate before they subsequently die (Wehner, 2020). Their behavioral repertoire comprises largely innate behaviors with little behavioral flexibility, a feature that applies to most ant species (Gronenberg, 2008). Gynes, on the other hand, aim at founding a new colony after mating, which requires advanced orientation abilities to be able to return to the newly founded nest. These task differences outlined above call for specific adaptations in sensory systems and high-order brain centers related to spatial orientation.

Both the interior–exterior transition and the period of learning walks at the beginning of the ants' foraging career are accompanied by plastic synaptic changes in neurocircuits of two visual pathways (Rössler, 2019; Wehner, 2020). The brain of *Cataglyphis* shows typical features or hymenopteran brains. Visual information from the compound eyes is processed via the optic lobes (OL), and two distinct pathways project to two sensory integration centers — the central complex (CX) and the mushroom bodies (MB) (Grob et al., 2017, 2019; Habenstein et al., 2020; Rössler, 2019; Schmitt et al., 2016). Panoramic visual information is stored in the MBs, centers for learning and memory, while directional information from the sky-compass network is fed into the CX via the bulbs of the lateral complex (LX) (Grob et al., 2019; Rössler, 2019). The ants' antennae house sensory structures for mechanosensory and olfactory information. Olfactory information is relayed to the glomeruli, odor processing units in the antennal lobes (ALs) and then also transferred to the MBs (Habenstein et al., 2020). Mechanosensory information is processed in the antennal mechanosensory and motor center (AMMC) and several integration centers in posterior brain neuropils (Grob, Tritscher, et al., 2021; Habenstein et al., 2020; Stieb et al., 2011).

Brain neuropil volumes can serve as a first proxy to look at adaptations to specific behavioral and orientation needs (Gronenberg, 2008). We therefore compared relative volumes of major neuropils and their substructures in primary and secondary brain centers important for spatial orientation and navigation (AL, OL, CX, and MB) of males, gynes and workers from the Mediterranean desert ant *Cataglyphis nodus*. We extended the analyses to the level of neurocircuits by comparing absolute numbers of synaptic complexes in two visual pathways to the MBs and CX. We hypothesize that the different lifestyles of *Cataglyphis* castes and sexes are reflected in specific neuronal adaptations for spatial orientation — while the female brain needs to be equipped for flexible navigation tasks with large centers for learning and memory, male brains should be more narrowly tuned to innate behaviors for finding a mate.

2 | METHODS

2.1 | Animals

Winged males (♂) (Figure 1(a)) and gynes (♀, virgin queens, Figure 1(b)) of *Cataglyphis nodus* (Brullé, 1832) were collected while leaving the nest entrance of two colonies over the course of 1 day at Kotychi-Strofylia National Park, Lapas, Greece in the summer of 2019. Additionally, worker ants (☿, Figure 1(c)) that brought food back to the colony (foragers) were collected at Schinias National Park, Marathonas, Greece. Ants were kept one night in darkness prior to dissection following the same procedure as established in Grob et al. (2017).

2.2 | Antibody characterization

To determine the volume of neuropils in the *Cataglyphis* brain, a monoclonal antibody to synapsin (SYNORF1, mouse@synapsin; kindly provided by E. Buchner and C. Wegener, University of Würzburg, Germany) was used to visualize synapse-rich neuropils (Table 1). Synapsin is highly conserved among invertebrates and present in presynaptic terminals. The specificity of the antibody has been characterized previously for *Cataglyphis* ants (Grob, Tritscher, et al., 2021; Habenstein et al., 2020; Schmitt et al., 2016, 2017; Stieb et al., 2010, 2012).

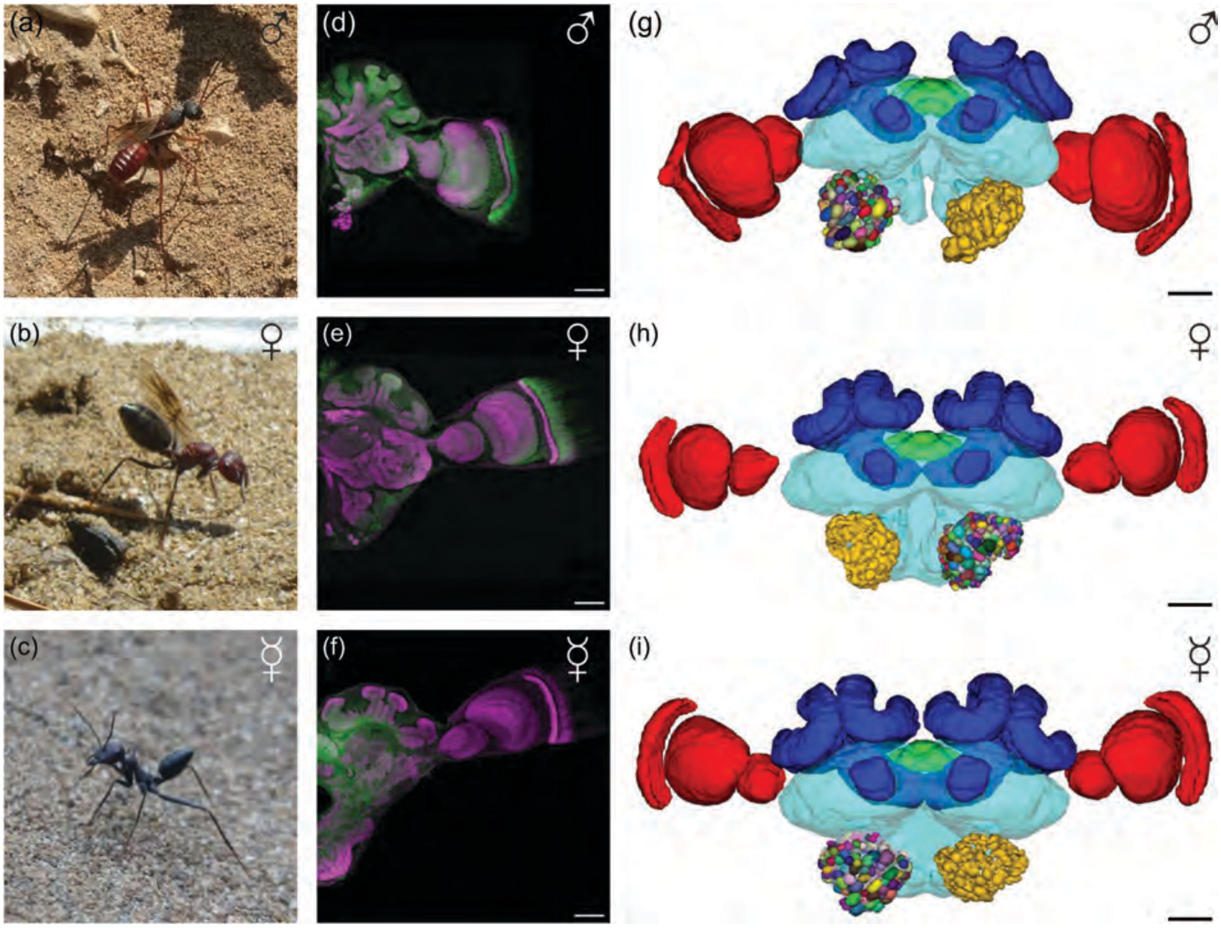

FIGURE 1 Phenotypes of males and the two female castes of *Cataglyphis nodus* and their brains. (a–c) The reproductive castes of *C. nodus* (a) males (♂) and (b) gynes (virgin queens, ♀) are winged and capable of flight, while (c) the infertile female workers do not have wings and walk (☿) [images taken from (Fleischmann et al., 2020a)]. (d–f) To compare the brains of (d) male, (e) gyne, and (f) worker ants, their brains were double labeled with anti-synapsin antibodies (magenta) and f-actin labeling with fluor-phalloidin (green) (only the left brain hemispheres are shown). (g–i) The 3D-reconstruction of the brains revealed significant differences between the brains of (g) male, (h) gyne, and (i) worker ants, specifically in the following neuropils of interest: Mushroom bodies (MB, blue), central complex (CX, green), optic lobes (OL, red), and antennal lobes (AL, yellow). The individual glomeruli of the AL of one side (side chosen pseudo-randomized) are shown multicolored. The protocerebral lobes and gnathal ganglia are shown in turquoise. Scale bars = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Antibody characterization

Antibody	Immunogen	Manufacturer; species; clonality; Cat #; RRID
Synapsin	Drosophila synapsin glutathione-S-transferase fusion protein	E. Buchner, Theodor-Boveri-Institute, University of Würzburg, Germany; mouse; monoclonal; Cat # 3C11 (SYNORF1); RRID: AB_528479

2.3 | Neuroanatomical procedures

Brains were double stained using a primary antibody to synapsin (SYNORF1, E. Buchner, University of Würzburg, Germany) and CF633 Phalloidin (00046, Biotium Inc., Fremont, California) using a previously established protocol (Habenstein et al., 2020).

For brain dissection, all experimental ants were anesthetized on ice. The ants were then decapitated and the brains were immediately dissected under cooled ringer solution (127 mM NaCl, 7 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.8 mM TES, and 3.2 mM trehalose, pH 7.0). The brains were transferred to a 64-well-plate and fixated in 4% formaldehyde phosphate-buffered saline (PBS) at 4°C overnight. Subsequently, the brains were rinsed in PBS, three times for 10 min each. To permeabilize cell membranes for antibody application on the whole-mount brains, the brains were rinsed once with 2% Triton-X 100 for 10 min and twice with 0.2% Triton-X 100 for 10 min. The brains were then incubated for 1 h at room temperature on a shaker in a 0.5% Triton-X 100 solution in PBS with 2% of Normal Goat Serum (NGS, Jackson ImmunoResearch Laboratories), to block unspecific binding sites. Then they were incubated on a shaker using a primary antibody to synapsin from mouse 1:50 in PBS with 0.5% Triton-X 100 and 2% NGS for 5 days in a fridge at 4°C. After incubation, the brains were rinsed three times in PBS for 20 min.

Subsequently, the brains were incubated in a secondary antibody coupled to AlexaFluor 568 (A12380, Molecular Probes, Eugene, Oregon) dye (1:250), and CF633 Phalloidin (2.5 μL Phalloidin from Methanol stock solution in 500 μL PBS) in PBS with 0.5% Triton-X 100 and 1% NGS for 3 days on a shaker at 4°C. The brains were rinsed in PBS, four times for 20 min each, and post-fixated in 4% formaldehyde in PBS overnight at 4°C. Afterward, they were washed four times in PBS for 20 min each and subsequently dehydrated in an ascending series of ethanol in water dilution (30%, 50%,70%, 90%, and 95% ethanol in water for 3–4 min each, and two times in 100% ethanol for 5 min). The brains were finally cleared in methyl salicylate (4529.1, Carl Roth Gmbh & Co. Kg, Karlsruhe, Germany).

2.4 | Data analyses

2.4.1 | Neuroanatomical analyses

A confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems GmbH, Wetzlar, Germany) was used for scanning the brains as image stacks at a step size of 5 μm. We used an APO 20×/0.7 IMM water immersion objective for overviews with 0.75 digital zoom for whole-brain scans, with 1.6 zoom for closeups of the CX, with 2.0 zoom for the MB calyx, and with 4.0 zoom for detailed scans of the LX. The PL APO 63×/1.2 W objective with 2.0 digital zoom was used for high-resolution scans in the lip (Li) and collar (Co) of the MB calyx.

The major neuropils and their subunits were easily distinguishable in anti-synapsin labeled whole mount brains (Figure 1(d–f)). Volumes of brain neuropils of interest were analyzed using the 3D-reconstruction software TrakEM2 (Cardona et al., 2012) plugin for ImageJ 1.52n (Wayne Rasband, National Institutes of Health, The United States).

To compare numbers of synaptic complexes between sexes and castes, microglomeruli (MG) were quantified with Amira (Amira-Avizo Software 2019.1, Thermo Fisher Scientific Inc., Waltham, Massachusetts) in visual and olfactory subregions of the MB calyx (Co, Li) using the AMIRA tracking tool following the protocol by Groh et al. (2012) [for further details see Rössler et al., 2017]. MG densities were analyzed by averaging multiple sample volumes, in the Li (two) and the Co (three) as numbers of MG per 1000 μm^3 following the procedure introduced by Groh et al. (2014). The total numbers of MG per neuropil were estimated by multiplying the densities with the volume of Li, and Co respectively. This represents a good approximation, as in *C. nodus* MG densities are largely homogeneous throughout the Li and Co neuropils (Grob et al., 2017; Stieb et al., 2010).

In the LX bulbs, numbers of synaptic complexes in one brain hemisphere were counted using the TrakEM2 (Cardona et al., 2012) plugin for ImageJ 1.52n (Wayne Rasband, National Institutes of Health, The United States) to trace individual synaptic complexes.

2.4.2 | Statistical analyses

For statistic comparison, we used a non-parametric Kruskal–Wallis-test ($\alpha = 0.05$), and post hoc Mann–Whitney U-test with Bonferroni correction ($\alpha = 0.05$, after Bonferroni correction: $\alpha_{\text{corrected}} = 0.0167$). Since the absolute brain volumes differed significantly between the sexes and castes (♂, ♀, and ☿), the proportion of each neuropil (OLs, MBs, CX, and ALs) was used to compare relative neuropil volumes. The volume of each neuropil was divided by the total volume of the brain (method adapted from Nishikawa et al., 2008). In the case of synaptic complexes, we compared absolute numbers in the MB Li, Co, and LX. Absolute numbers of AL glomeruli were also compared statistically. Since the total numbers of glomeruli and AL sizes differed significantly between sexes and castes, relative measurements of glomerular volumes were used to compare AL glomeruli. For each AL, the median of glomerular volumes was calculated. By dividing the glomerular volume by the median, the relative volume of each glomerulus was calculated [method adapted from Stieb et al., 2011]. The relative volumes show how much larger the volume of a glomerulus is compared with the median size of all glomeruli. Outliers were defined as glomeruli with a higher value than 1.5 times the interquartile range of the 25th and 75th percentiles of the ALs. All statistical analyses were done with MATLAB (2015a or 2018a, The MathWorks Inc., Natick, Massachusetts).

2.5 | Nomenclature

We refer to Habenstein et al. (2020) (see also https://www.insectbraindb.org for 3D data of the *Cataglyphis* brain) and Ito et al. (2014) for the nomenclature of neuropils in the ant brain. For terms related to spatial orientation and navigation, we refer to Grob, el Jundi, and Fleischmann (2021).

3 | RESULTS

While the different sexes and castes in *C. nodus* have vastly different tasks and locomotory demands (flying, ambulatory), we found that the overall structure of their brains is remarkably similar (Figure 1(d–i)). The total brain volumes, including OLs, ALs, MBs, CX, protocerebral lobes, and gnathal ganglia, differ markedly between the sexes. Male *C. nodus* (median ± median absolute deviation [MAD]: $(58.0 \pm 5.51) \times 10^6\ \mu m^3$) have a significantly smaller brain volume compared with females, both gynes (median ± MAD: $(85.2 \pm 0.91) \times 10^6\ \mu m^3$) and workers (median ± MAD: $(78.8 \pm 6.19) \times 10^6\ \mu m^3$) (Kruskal–Wallis test: Total Brain Volume: $\chi^2 = 8.97$; $n = 14$; $p = .0113$; Mann–Whitney *U*-test with Bonferroni correction [$n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 5$]: ♂ vs. ♀ $p = .0159$; ♂ vs. ☿ $p = .0159$; ♀ vs. ☿ $p = .222$; Figure 2). The total brain size of workers and males scatter more than the brain volumes of gynes.

3.1 | Relative volumes of neuropils

Since the total brain volume differed significantly between the sexes and there might be size-dependent differences within the groups, subsequent analyses used relative neuropil volumes to compare between sexes and castes (for absolute volumes of the neuropils and their variation within and across groups, see Table 2). Despite the small sample sizes ($n_{♂} = 4$; $n_{♀} = 5$; and $n_{☿} = 5$), the results show significant differences between sexes and castes.

The OLs of males are relatively larger compared with those in both female castes (median ± MAD: ♂ = (26.78 ± 2.31)%; ♀ = (16.15 ± 0.70)%; ☿ = (14.27 ± 2.07)%; Kruskal–Wallis test: relative volume OLs: $\chi^2 = 8.97$; $n = 14$; $p = .0113$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 5$): ♂ vs. ♀; $p = .0159$; ♂ vs. ☿ $p = .0159$; ♀ vs. ☿ $p = .222$; Figure 3(a)).

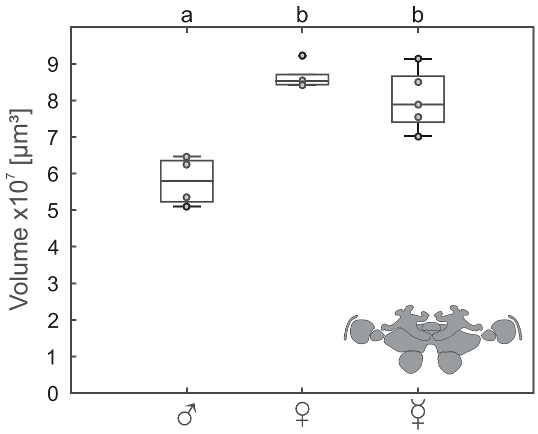

FIGURE 2 Total brain volume of male (♂, $n = 4$), gyne (♀, $n = 5$), and worker (☿, $n = 5$) *Cataglyphis nodus* ants. The central line of each boxplot depicts the median of the data. The upper and lower limits of the boxes show the 25th and 75th percentiles, while the whiskers extend to the extreme data points without outliers. Additionally, all data points are plotted as gray circles. Significant differences between the groups are marked with lower case letters (Kruskal–Wallis test and post hoc Mann–Whitney *U*-test with Bonferroni correction (after correction $p < .0167$)). Plotted neuropiles are filled in the brain diagram. For statistical details, see text

The MB volumes are smallest in males and largest in female workers (median ± MAD: ♂ = (21.08 ± 0.74)%; ♀ = (25.46 ± 0.35)%; ☿ = (33.64 ± 0.59)%; Kruskal–Wallis test: relative volume MBs: $\chi^2 = 11.57$; $n = 14$; $p = .00307$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 5$): ♂ vs. ♀ $p = .0159$; ♂ vs. ☿ $p = .0159$; ♀ vs. ☿ $p = .00794$; Figure 3(b)).

While the relative volumes of the CX do not differ between the female castes, the relative CX volumes are significantly larger in males (median ± MAD: ♂ = (1.25 ± 0.07)%; ♀ = (1.06 ± 0.00)%; ☿ = (1.09 ± 0.00)%; Kruskal–Wallis test: relative volume CX: $\chi^2 = 8.46$; $n = 14$; $p = .0145$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 5$): ♂ vs. ♀ $p = .0159$; ♂ vs. ☿ $p = .0159$; ♀ vs. ☿ $p = .421$; Figure 3(c)).

The AL relative volumes were found largest in males, while the smallest relative volumes were measured in workers (median ± MAD: ♂ = (5.67 ± 0.71)%; ♀ = (3.72 ± 0.14)%; ☿ = (2.28 ± 0.15)%; Kruskal–Wallis test: Relative Volume ALs: $\chi^2 = 11.57$; $n = 14$; $p = .00307$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 5$): ♂ vs. ♀ $p = .0159$; ♂ vs. ☿ $p = .0159$; ♀ vs. ☿ $p = .00794$; Figure 3(d)).

3.2 | Organization of antennal lobes

The overall AL structure characterized by its glomerular organization was found similar across sexes and castes (Figure 4), but the number of glomeruli in the AL of males (median ± MAD: 198 ± 2) is significantly lower compared with both female castes (median ± MAD: ♀: 245 ± 0; ☿: 250 ± 1) (Kruskal–Wallis test: number of glomeruli in the AL: $\chi^2 = 9.85$; $n = 14$; $p = .00730$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 5$): ♂ vs. ♀ $p = .0159$; ♂ vs.

TABLE 2 Absolute volumes of the neuropils in μm^3

Absolute volumes in μm^3	Total brain	Optic lobes	Mushroom bodies (MBs)	MB collar	MB lip	Central complex	Antennal lobes
Male 1	64,623,296	16,408,479	10,840,396	1,901,354	5,339,490	793,542	4,039,227
Male 2	62,431,130	19,484,811	13,089,777	2,254,900	6,430,775	789,091	3,178,627
Male 3	50,949,617	12,002,449	11,441,079	1,785,094	5,991,816	726,655	3,916,246
Male 4	53,598,907	15,102,832	11,354,829	2,075,398	5,512,066	607,677	2,588,787
Gyne 1	85,241,652	15,104,644	21,704,108	3,359,623	10,722,992	799,604	3,283,202
Gyne 2	85,349,570	14,541,797	23,604,343	4,019,076	12,297,651	904,033	3,156,365
Gyne 3	84,331,499	13,079,062	21,754,022	3,596,508	11,207,233	901,657	2,778,856
Gyne 4	92,185,437	14,241,097	23,145,185	3,377,755	12,017,572	1,009,589	3,425,313
Gyne 5	84,183,384	13,596,244	19,639,564	3,247,239	9,869,060	895,655	3,395,878
Worker 1	78,834,648	9,620,570	26,518,635	4,879,379	13,583,573	792,951	1,545,798
Worker 2	91,317,985	15,122,095[a]	31,715,744	4,804,828	15,917,356	999,193	2,118,880
Worker 3	85,027,529	12,132,646	27,323,110	4,513,799	13,389,884	925,384	2,065,849
Worker 4	75,324,271	12,401,030	25,714,441	4,352,230	13,398,593	812,843	1,716,782
Worker 5	70,207,013	9,466,550	23,196,240	3,990,656	11,745,636	768,341	1,193,527

[a] One optic lobes was missing in this preserved brain. Thus, the remaining optic lobe was doubled for the total volume.

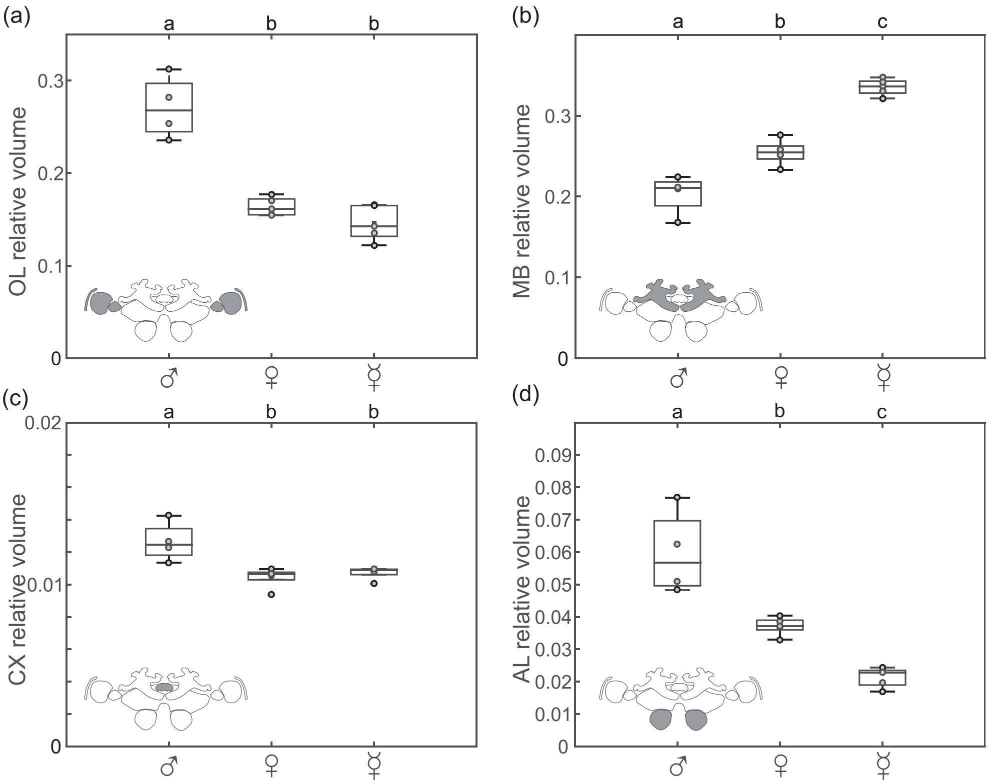

FIGURE 3 Relative volumes of neuropils of interest. (a) Optic lobes (OL), (b) mushroom bodies (MB), (c) central complex (CX), and (d) antennal lobes (AL) in male (♂, $n = 4$), gyne (♀, $n = 5$), and worker (☿, $n = 5$) ants. Plotted neuropiles are gray in the brain diagram. For figure conventions, see Figure 2, and for statistical details, see text

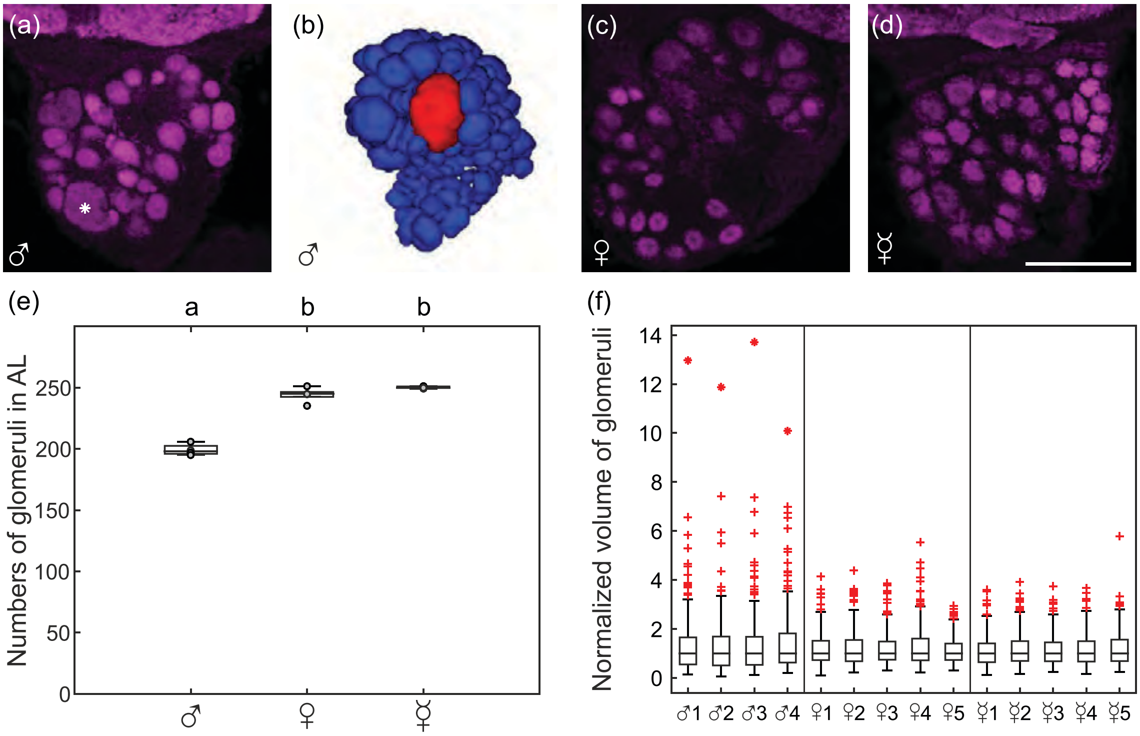

FIGURE 4 Numbers and relative sizes of antennal lobe (AL) glomeruli in male (♂, $n = 4$), gyne (♀, $n = 5$), and worker (☿, $n = 5$) ants. (a,c,d) Labeling with anti-synapsin antibody (magenta) of the AL glomeruli of (a) males, (c) gynes, and (d) workers. Highly enlarged macroglomerulus is marked with an asterisk (view from frontal). (b) The highly enlarged macroglomerulus is highlighted (red) in the 3D reconstruction of the male AL glomeruli (blue) (view from dorsal). (e) Numbers of enlarged glomeruli in the ALs. (f) Normalized volumes of the glomeruli. Outliers are shown as red crosses, with significant outliers highlighted as an asterisk. For figure conventions, see Figure 2, and for statistical details, see text [Color figure can be viewed at wileyonlinelibrary.com]

☿ $p = .0159$; ♀ vs. ☿ $p = .0952$; Figure 4(e)). Only the ALs of males contain highly enlarged glomeruli (so called macroglomeruli) exceeding more than 10 times the median size of the glomeruli in the ALs (Figure 4(f)).

3.3 | Synaptic complexes in the mushroom bodies and lateral complex

Comparison of the absolute numbers of synaptic complexes (microglomeruli, MG) in the MB calyx revealed significant differences between the sexes and castes (Table 3). The estimated total numbers of MG in the MB Co are significantly lower in males (median ± MAD: 195,542 ± 18,107) than in female *C. nodus* (median ± MAD: ♀: 397,614 ± 23,272; ☿: 394,864 ± 34,211) (Kruskal–Wallis test: Number of MG in the MB Co: $\chi^2 = 8.72$; $n = 17$; $p = 0.0128$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 8$): ♂ vs. ♀ $p = .0159$; ♂ vs. ☿ $p = .0159$; ♀ vs. ☿ $p = .833$; Figure 5(a)). The density of Co MG is significantly higher in gynes compared with workers, but we found no statistical difference between males and both female castes (Kruskal–Wallis test: density of MG in the Co: $\chi^2 = 9.63$; $n = 17$; $p = .00812$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 8$): ♂ vs. ♀ $p = .0635$; ♂ vs. ☿ $p = .299$; ♀ vs. ☿ $p = .00155$; Figure 5(b)).

In the MB Li the pattern is similar to the one found in the Co. Males have significantly smaller numbers of MG in the Li than both female castes (median ± MAD: ♂: 282,629 ± 15,316; ♀: 751,819 ± 44,536; ☿: 695,431 ± 81,293) (Kruskal–Wallis test: number of MG in the Li: $\chi^2 = 9.29$; $n = 17$; $p = .00960$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 8$): ♂ vs. ♀ $p = .0159$; ♂ vs. ☿ $p = .00404$; ♀ vs. ☿ $p = .354$; Figure 5(c)). MG are packed more densely in the Li of gynes compared with both males and workers (Kruskal–Wallis test: density of MG in the MB Li: $\chi^2 = 10.14$; $n = 17$; $p = .00627$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 5$; $n_{☿} = 8$): ♂ vs. ♀ $p = .0159$; ♂ vs. ☿ $p = .651$; ♀ vs. ☿ $p = .00155$; Figure 5(d)).

In the LX, the number of synaptic complexes (giant synapses, GS) was lowest in males (median ± MAD: 88.5 ± 1.5) and slightly higher counts were found in workers (median ± MAD: 95 ± 4) and gynes (median ± MAD: 103 ± 0.5). No statistical difference was found between the sexes and castes after Bonferroni correction (Kruskal–Wallis test: Number of GSs in the LX: $\chi^2 = 10.18$; $n = 14$; $p = .00615$; Mann–Whitney *U*-test with Bonferroni correction ($n_{♂} = 4$; $n_{♀} = 4$; $n_{☿} = 5$): ♂ vs. ♀ $p = .0286$; ♂ vs. ☿ $p = .0317$; ♀ vs. ☿ $p = .0317$; Figure 5(e)).

4 | DISCUSSION

4.1 | Polymorphisms in olfactory neuropils

Due to their special role in eusocial insect colonies, the behavioral repertoires of males are focused on few specific tasks, namely finding a virgin queen and mating. It has previously been suggested, that in *Cataglyphis fortis* sex pheromone communication plays an important role during courtship and mating behavior (Stieb et al., 2011). The brains of hymenopteran males usually reflect this specialization. In the AL the number of glomeruli in males is largely

TABLE 3 Number of synaptic complexes in mushroom bodies and the lateral complex and number of glomeruli in the antennal lobes

	Microglomeruli in MB collars	Microglomeruli in MB lips	Giant synapses in one lateral complex	Glomeruli in one antennal lobe
Male 1	205,452	275,874	91	199
Male 2	233,006	345,654	89	206
Male 3	185,633	289,383	78	197
Male 4	169,238	258,751	88	195
Gyne 1	420,886	665,719	103	245
Gyne 2	439,866	758,355	103	245
Gyne 3	397,614	751,819	–	245
Gyne 4	339,652	871,274	109	251
Gyne 5	384,257	707,283	102	235
Worker 1	460,830	747,096	101	250
Worker 2	377,713	716,281	102	249
Worker 3	437,588	792,235	–	251
Worker 4	425,551	770,419	93	251
Worker 5	379,112	592,176	95	250
Worker 6	325,275	607,834	91	–
Worker 7	337,239	537,762	–	–
Worker 8	415,606	674,581	–	–

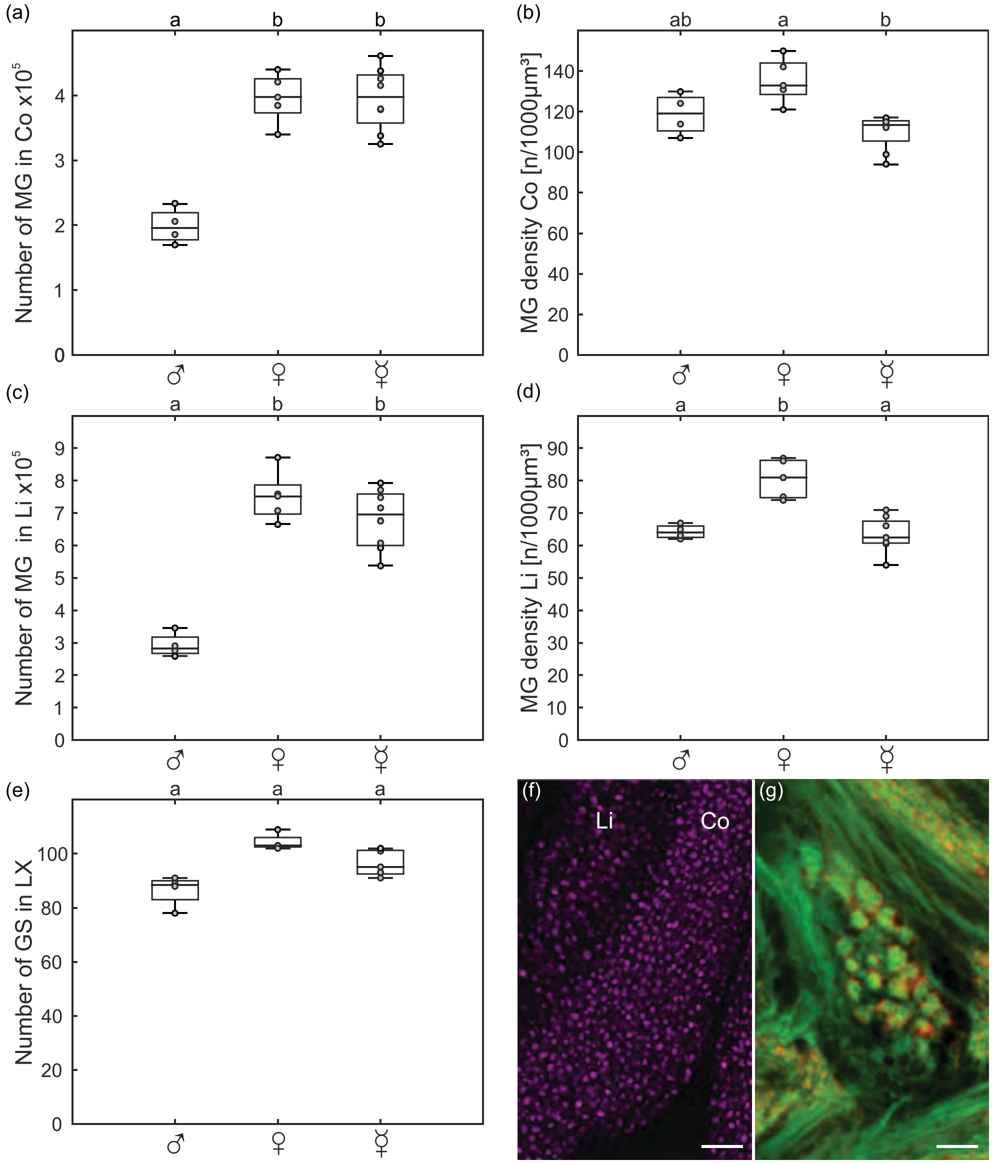

FIGURE 5 Differences in the total numbers and densities of synaptic complexes in the mushroom bodies (MBs) and lateral complex (LX). (a–d) Microglomeruli (MG) in MB calyx subdivisions in males (♂, $n = 4$), gynes (♀, $n = 5$), and workers (☿, $n = 8$). (a) Total number of MG in the visual input region of the MB, the collar (Co). (b) Density of MG in the Co. (c) Total number of MG in the olfactory input region of the MBs, the lip (Li). (d) Density of MG in the Li. (e) Total number of giant synapses (GS) in the LX in males (♂, $n = 4$), gynes (♀, $n = 4$), and workers (☿, $n = 5$). For figure conventions, see Figure 2, and for statistical details, see text. (f,g) Confocal images of synaptic complexes in (f) the MB (☿), and (g) the LX (♀). The synaptic complexes are labeled with an antibody to the presynaptic protein synapsin (magenta). In the LX, double staining with f-actin by fluor-phalloidin (green). Scale bars = 10 µm [Color figure can be viewed at wileyonlinelibrary.com]

reduced compared with females. This is true for desert ants like *C. nodus*, or *C. fortis* (Stieb et al., 2011), but also for other ants like *Atta vollenweideri* (Kuebler et al., 2010), *Camponotus floridanus* (Zube & Rössler, 2008), *Camponotus japonicus* (Nishikawa et al., 2008), and *Harpegnathos saltator* (Hoyer et al., 2005), as well as for the honey bee *Apis mellifera* (Arnold et al., 1985; Brockmann & Robinson, 2007). The number of AL glomeruli between the female castes of *C. nodus* does not differ. This is different in *C. fortis*, where queens have more AL glomeruli than workers (Stieb et al., 2011). In this case, *C. nodus* is more similar to other ant species, where the number of AL glomeruli in queens are comparable to those of workers (Kuebler et al., 2010; Nishikawa et al., 2008). Since many olfactory tasks involved in the maintenance of the colony are solely performed by females (Hölldobler & Wilson, 1990), a higher number of glomeruli is needed, for example for brood and nest related communication. In absolute numbers, the numbers of glomeruli in the ALs of *C. nodus* exceed those found in *C. fortis* (Stieb et al., 2011), but was also found to be smaller than in other ant species less specialized for visual navigation (Kuebler et al., 2010; Nishikawa et al., 2008; Zube & Rössler, 2008).

While *C. nodus* males have the lowest number of glomeruli in the ALs, they have a larger relative volume of ALs compared with females. This is in line with the results found in *C. japonicus* (Nishikawa et al., 2008). The large size of male ALs is mainly due to massively enlarged individual macroglomeruli. The occurrence of macroglomeruli has been described for males of several ant species, like *A. vollenweideri* (Kuebler et al., 2010), *C. fortis* (Stieb et al., 2011), *C. japonicus* (Nishikawa et al., 2008), and *H. saltator* (Hoyer et al., 2005). In a variety of insects, macroglomeruli are exclusively receiving input from sex-pheromone-sensitive olfactory receptor neurons [e.g., *Manduca sexta* (Hansson et al., 1991), *A. mellifera* (Arnold et al., 1985)]. It has been previously suggested, that pheromone communication plays an important role in the mating behavior of *Cataglyphis* (Stieb et al., 2011). However, thus far, only very little is known about *Cataglyphis'* mating behavior and the use of sex pheromones (Wehner, 2020). Large numbers of olfactory receptor neurons converge and terminate in macroglomeruli, largely contributing to their increased volume (Berg et al., 1998; Vickers & Baker, 1997). The results in *C. nodus* demonstrate that the structural organization of the ALs expresses both sexual-dimorphism (macroglomeruli are present exclusively in males) and caste-specific polymorphism (difference in the relative volumes of the ALs).

Olfactory information leaves the AL via two olfactory pathways to the Li of the MB and the lateral horn (LH), the medial and lateral antennal lobe tracts (ALTs) (Habenstein et al., 2020). Compared with females, the medial ALT is reduced in males of *C. floridanus* (Zube & Rössler, 2008). In the honeybee, the lateral ALT does not show learning-dependent plasticity (Peele et al., 2006). It has been suggested, that the reduction of the medial ALT in male *Camponotus* ants may reflect the dominance of less plastic olfactory circuits to higher brain centers (Zube & Rössler, 2008). Male *C. nodus* have the relatively smallest MBs and workers the largest, which is reflected in about twice the absolute numbers of MB calyx synaptic complexes in the Li and Co. Between female ants, the total number of synaptic complexes does not differ significantly, although the MB volume is smaller in gynes. Interestingly, gynes make up for their smaller MBs with an increased synaptic density, mainly in the Li. The MG density has previously been shown to be an intraspecies limiting factor in the neuronal architecture of the brain in leaf-cutting ant worker castes (Groh et al., 2014). The high numbers of synaptic complexes in the MB calyx Li and of glomeruli in the ALs emphasize the importance of olfaction-related behavioral tasks in both female castes of *Cataglyphis*. In contrast, the male olfactory neuropils reflect fewer and more specific olfactory tasks like finding a gyne, potentially by olfactory non-compass orientation based on sex pheromone communication.

4.2 | Polymorphisms in visual neuropils

Visual information is transferred via visual pathways from the OLs into high-order integration centers in the ant brain (Grob et al., 2017; Habenstein et al., 2020; Rössler, 2019; Schmitt et al., 2016). In *C. nodus*, we found a sexual dimorphism in the relative size of the OLs. Males possess larger OLs compared with both female castes. Similar results have been found in *A. vollenweideri* (Kuebler et al., 2010) and *C. japonicus* (Nishikawa et al., 2008). Large optic lobes correlate with high amounts of sensory input. Likewise, more ommatidia in the compound eye allow a better spatial resolution. A higher number of ommatidia in male ants compared with their female conspecifics is common among ants (Gronenberg, 2008), indicating that vision is an important cue for males to find mates. In some species, workers may possess even fewer ommatidia than gynes (Narendra et al., 2016). In some insects males even have specialized region that is tuned for small field motion detection, so-called “love spots,” in their eyes (Perry & Desplan, 2016). It will be interesting in future work to analyze how differences and similarities in ommatidia numbers and organization are reflected in the numbers and volumes of cartridges and columns in the optic lobes.

In *C. nodus*, one obvious distinction between the non-reproductive female worker caste and the reproductive caste is that workers are purely ambulatory, while males and gynes are winged and capable of flight. This difference in locomotion might also lead to different neuronal needs for spatial orientation and navigation. Flight might lead to the need for a higher temporal visual resolution. Like the ants' eyes, also other sensory organs are adapted to the different types of locomotion. One example is the mechanosensory Johnston's organ (JO) in the ants' antennae that is involved in several navigational tasks including wind-compass orientation, and flight control. Interestingly, the number of JO sensory neurons is higher in the alates than in *C. nodus* workers (Grob, Tritscher, et al., 2021).

In *Cataglyphis*, visual information in general is an important navigational cue for the ants (Wehner, 2020). Information from polarized skylight is projected via a highly conserved pathway from the OLs via the anterior optic tract and several stages to the LX to finally terminate in the CX (Grob et al., 2017, 2019; Schmitt et al., 2016). Our present study, for the first time in any insect, shows that the number of large synaptic complexes does not differ between males and the two female castes. These giant synapses form an important relay station of the sky-compass pathway and have been proposed to play an important role in the processing of sky-compass relevant cues by allowing for a fast and reliable signal transmission along this specialized visual pathway (Held et al., 2016).

The CX is a highly conserved neuropil (Homberg, 2008) that plays a crucial role in integration of orientation cues and motor output (Honkanen et al., 2019). Our results revealed that while the absolute volume of the CX is smallest in males, the relative size of the CX in males is larger than in females. This suggests the importance of neuronal circuits for high-order integration of sensory stimuli relevant for spatial orientation and locomotion control, even in short-lived males, most likely due to the necessity for promoting the efficacy of male mating behavior. This sex-specific dimorphism in CX volume is consistent with results from *A. vollenweideri* (Kuebler et al., 2010) and *C. japonicus* (Nishikawa et al., 2008). To better address the smaller absolute size of the CX in males, synaptic counts within subunits of the CX would be helpful in the future. However, this was not possible with confocal imaging techniques as anti-synapsin labeled neuropil is very dense in this region. Therefore, we decided to use relative CX volumes as a suitable first proxy to look at neuronal adaptations (Gronenberg, 2008).

Information about the visual scenery (panorama) is transferred from a retinotopical organization in the OLs to the MB Co via the anterior superior optic tract (Grob et al., 2017, 2019; Schmitt et al., 2016). The MBs are essential for learning-based orientation, like visual landmark orientation (Buehlmann et al., 2020; Kamhi et al., 2020). Comparable to conditions in the olfactory compartments (Li), the numbers of synaptic complexes in the Co are significantly smaller in males but show no difference between the female castes. The vast numbers of synaptic complexes and the associated parallel MB microcircuits in *Cataglyphis* workers are well suited for storing visual images, which can be used for orientation based on view memories (Ardin et al., 2016; Grob et al., 2017). Foraging ants need large computational capacities for learning the visual panorama, routes, and landmarks to navigate successfully back to their nest every time they leave it. Additionally, foragers rely on a time-compensated sun compass during their long foraging runs, for which they have to learn and memorize the course of the sun over the day, potentially in relation to panoramic features, whenever available (Wehner & Lanfranconi, 1981; Wehner & Müller, 1993). In *Cataglyphis* species that show mating swarm behavior, mated queens find their new colony alone, without the help of workers (Boulay et al., 2017; Peeters & Aron, 2017). Thus, young queens face similar navigational challenges as workers do and need to learn the position of their newly founded nest. Males, in contrast, have small MBs. They do not need to learn or remember the location of a nest, since they will never return to a nest after leaving their natal colony (Boulay et al., 2017; Hölldobler & Wilson, 1990; Peeters & Aron, 2017). Male ants of all examined species thus far possess a large CX that is comparable in size to the CX of workers (Gronenberg, 2008). The voluminous OLs together with a large CX and a small synaptic capacity in the MB of *C. nodus* males suggest the requirement for a high degree of specification and precision during visual orientation at the expense of behavioral flexibility, most importantly learning-related changes in behaviors.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

Robin Grob: Conceived the study, collected the data, analyzed the data, visualized the data, drafted. **Niklas Heinig:** Collected the data, analyzed the data. **Kornelia Grübel:** Analyzed the data. **Wolfgang Rössler:** Conceived the study, led the study, revised the manuscript. **Pauline N. Fleischmann:** Conceived the study, led the study, analyzed the data, visualized the data, revised the manuscript. All authors approved the final version of the manuscript for submission.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

All data are supplied within the manuscript and supplement. Raw confocal microscopy images and 3D data are available from the authors upon reasonable request.

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6 Manuscript 5: Rotation of Skylight Polarization During Learning Walks is Necessary to Trigger Neuronal Plasticity in *Cataglyphis* Ants

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Rotation of skylight polarization during learning walks is necessary to trigger neuronal plasticity in *Cataglyphis* ants

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Many animals use celestial cues for impressive navigational performances in challenging habitats. Since the position of the sun and associated skylight cues change throughout the day and season, it is crucial to correct for these changes. *Cataglyphis* desert ants possess a time-compensated skylight compass allowing them to navigate back to their nest using the shortest way possible. The ants have to learn the sun's daily course (solar ephemeris) during initial learning walks (LW) before foraging. This learning phase is associated with substantial structural changes in visual neuronal circuits of the ant's brain. Here, we test whether the rotation of skylight polarization during LWs is the necessary cue to induce learning-dependent rewiring in synaptic circuits in high-order integration centres of the ant brain. Our results show that structural neuronal changes in the central complex and mushroom bodies are triggered only when LWs were performed under a rotating skylight polarization pattern. By contrast, when naive ants did not perform LWs, but were exposed to skylight cues, plasticity was restricted to light spectrum-dependent changes in synaptic complexes of the lateral complex. The results identify sky-compass cues triggering learning-dependent versus -independent neuronal plasticity during the behavioural transition from interior workers to outdoor foragers.

1. Background

Finding the way back home is a crucial challenge that many animals face. Desert ants of the genus *Cataglyphis* use an impressive set of navigational cues to get back to their nest even under exceptionally harsh conditions. To spend as little time as possible in the blazing heat of the sun, *Cataglyphis* foragers steer back to their nest in the shortest way possible with astonishing accuracy [1]. The ants integrate directional information from a celestial compass and distance information from a step integrator into a vector pointing homewards [1]. However, a compass based on celestial cues comes with its own challenges. Both the position of the sun and the associated sky polarization pattern change throughout the day. The ant's internal skylight compass must compensate for this movement, especially during extended foraging trips, or upon subsequent visits to a profitable food site. This is especially important around solar noon when the sun's horizontal position (azimuth) changes most rapidly (figure 1). The sun's daily course (solar ephemeris) depends on the season and geographical position, making the problem even more complex. Due to these unpredictable variables, the inherent knowledge about the solar ephemeris is very limited [2]. Hence, a celestial compass must be learned before embarking upon far-ranging foraging journeys [2–5]. Desert ants perform well-structured initial learning walks (LW) during the transition phase

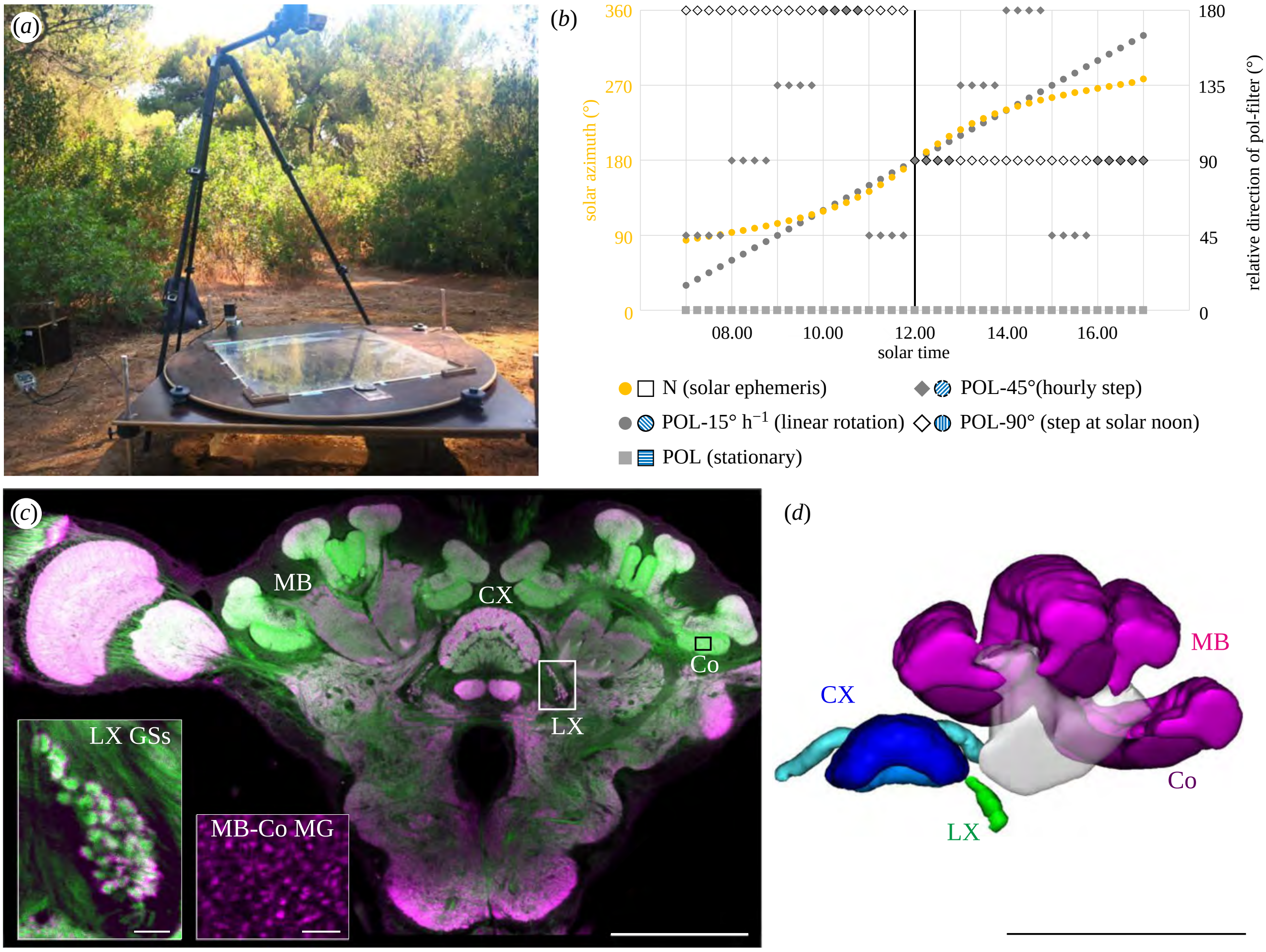

Figure 1. Experimental procedure. (*a*) A rotating table was installed 30 cm above the natural nest entrance of *Cataglyphis nodus*. In the opening in the middle of the table, a linear polarization filter was placed. Novices that were allowed to perform LW did so under the restricted area (60 × 60 cm) that was covered by the filter. (*b*) The linear polarization filter (POL) was rotated over the day in relation to the natural rotation of the sky polarization pattern (N, yellow circles (for the Schinias National Park on the 1st of August 2019)). The filter was either turned once a day at solar noon (POL-90°, white diamond), hourly (POL-45°, grey diamond) or continuously (POL-15° h^{-1}, grey circles), or was stationary over the day (POL, grey square). (*c*) Brains of ants that participated in the field experiments were double labelled with anti-synapsin antibodies (magenta) and f-actin labelling with fluor-phalloidin (green) (Scale bar, 200 µm). In this study, we focused on neuropils along with two major visual pathways: the central complex (CX), the mushroom body (MB) collar (Co) microglomeruli (MG) (MB-Co MG; black box and inset) and giant synapses in the lateral complex (LX GS; white box and inset). Scale bar insets = 10 µm. (*d*) Three-dimensional reconstruction of MB (magenta) with the Co in dark magenta cut open to highlight the lateral-complex structures (green) located posterior to the peduncle. CX in shades of blue (Scale bar, 200 µm). (Online version in colour.)

from interior worker to outside forager [6,7]. *Cataglyphis nodus* repeatedly stop their forward movement to perform characteristic turns [6] and stops with views directed towards their nest entrance. During this behaviour, naive ants do not rely on the celestial compass [4], but rather use the earth's magnetic field as directional input for path integration [8]. Full body turns have been suggested to be used to read out the celestial compass [6], for example by taking skylight snapshots [9]. Here, we investigate how the rotation of the sky influences neuroplasticity during the initial LW of *C. nodus*. Previous studies have shown that initial LW trigger structural synaptic plasticity in two visual pathways to integration centres in the ant brain [3,4,10]. The central complex (CX) [11] and mushroom bodies [12,13] are essential neuropils involved in memory-based orientation and navigation [14]. The CX has been suggested to be the neuronal correlate for path integration [15], whereas the mushroom bodies circuits have the capacity for storing view-based visual information [16].

In the present study, we ask whether the early experience of changing sky polarization patterns represents the necessary cue to induce volumetric and structural synaptic changes (neuronal plasticity) in integration centres along two major visual pathways. The results demonstrate that the experience of a rotating sky polarization pattern during the performance of initial LW in the natural habitat is necessary to trigger calibrations in neuronal circuits of the CX and mushroom bodies. This suggests that the natural rotation of the sky polarization pattern has to be acquired during this early learning phase.

2. Methods

(a) Experimental model and subject details

Four ant colonies of *Cataglyphis nodus* (Brullé 1832) in the pine forest of Kotychi-Strofylia National Park, Lapas, Greece and Schinias National Park, Marathonas, Greece were used in the summer of 2019 for this study. All ants outside the nest were marked with car paint for 3 consecutive days before the experiments started (Motip Lackstift Acryl, MOTIP DUPLI GmbH, Haßmersheim, Germany). This made sure that only unmarked novices that had not performed LW before the experiments were included in the experiments [17].

(b) Experimental procedure

(i) Light exposure

Interior workers were collected from two colonies that were excavated at night under a tent using red light in the Kotychi-Strofylia National Park (Strofylia-1, Strofylia-2); for more details see electronic supplementary material). All foragers of the colonies had been marked over 3 consecutive days beforehand. The ants were kept in a dark box until the next day. Interior workers (unmarked ants) were apportioned to four experimental groups in different boxes. Each group was exposed to light pulses under the natural sky five times a day (07.30, 09.30, 11.30, 13.30, 15.30 solar time) for 45 min each for 3 consecutive days (method established by Schmitt *et al*. [18]). The groups were exposed to different light conditions: full wavelength spectrum of natural sunlight under a UV-light permeable Plexiglas (Plexiglas (GS) 2458, Evonik Performance Materials GmbH, Essen, Germany) (LE-N), full wavelength spectrum of natural skylight with a stationary linear polarization pattern under a UV-light permeable linear polarizer (POL, OUV6060-C-HNP'B, Knight Optical Ltd, Harrietsham, UK) (LE-POL), natural skylight without light of wavelengths shorter than below 420 nm using a UV cut-off filter (Plexiglas (Gallery) 0A570 GT, Evonik Performance Materials GmbH, Essen, Germany) (LE-UVB) and no light within the dark box as a control (DD). All four boxes were otherwise treated identically. After the third day of exposure, the ants were kept in the dark overnight and were subsequently dissected, and the neuroanatomical staining process was started immediately in Greece (see below). This allowed for all groups to be stained at the same time after their experimental treatment.

(ii) Learning walk manipulations

Naive ants (novices) from two colonies (Strofylia-3 (figure 2), Schinias-1 (figure 3); for more details see electronic supplementary material) were allowed to perform LW in a restricted area around their natural nest entrance (60 × 60 cm restricted by a transparent plastic fence). Only experienced foragers (marked ants) that did not participate in the experiments were allowed to leave the fenced area. The spectrum of skylight the ants were able to perceive during their LW was manipulated with a linear, UV-light permeable polarization filter (60 × 60 cm, figure 1*a*). It was installed in the middle of a custom-made rotating table 30 cm above the nest entrance from the third day of marking. This made sure that ants that did not yet leave the nest but spent time inside the nest entrance could only perceive the experimental condition. To test whether changes in the polarization pattern over the course of the day for a sequence of 3 days were necessary to trigger neuronal plasticity, ants were presented with one of four POL rotations: (1) stationary POL, (2) 90° rotation of POL at solar noon, (3) rotation of POL by 45° every 60 min or (4) a continuously rotating POL at a speed of 15° h^{-1} (figure 1*b*). For the continuous rotation, the rotating table was moved by a stepper motor (Nema 17 stepper motor, stepperonline Inc., New York City, NY, USA) with a micro-stepping controller (SMC11, Nanotec Electronic GmbH & Co. KG, Feldkirchen, Germany). The stepper motor was controlled by an Arduino microcontroller (Genuino ZERO, arduino.cc) using a custom-written script and powered by a customized battery power supply made by the Biocentre's electronic workshop [8]. As a control, ants were allowed to perform LW under natural skylight, including the naturally moving polarization pattern. For both experiments (light exposure (LE, figure 2) and LW under a rotation polarizer (figure 3)), LW control groups under stationary POL (LW-POL-1 (in Strofylia), LW-POL-2 (in Schinias)) and under natural skylight (LW-N-1 (in Strofylia), LW-N-2 (in Schinias)) were collected. Novices were allowed to perform LW under one of these conditions for 3 consecutive days. During the LW, the ants were able to see the position of the sun. On the third day, ants that reached the fence, indicating that they had performed multiple LW previously [10], were caught and kept in a dark box until dissection the next day (method established by Grob *et al*. [4]). Subsequently, all ants outside of the nest were marked again for 3 consecutive days.

(c) Neuroanatomical procedures

To analyse volumetric and synaptic changes following LE and LW, the ants' brains were double labelled in the field laboratory in Greece using a primary antibody against synapsin (SYNORF1, kindly provided by E. Buchner, University of Würzburg, Germany) and CF633 Phalloidin (00046, Biotium Inc., Fremont, CA, USA) following established protocols from Habenstein *et al*. [19].

The ants were anaesthetized on ice, decapitated, and the brains were immediately dissected under cooled Ringer's solution (127 mM NaCl, 7 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.8 mM TES and 3.2 mM trehalose, pH 7.0). The brains were then transferred to a 24-well plate and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) at 4°C overnight. The brains were rinsed in PBS three times for 10 min each. To make the cell membranes permeable for antibody application, the brains were rinsed once with 2% Triton-X 100 for 10 min and twice with 0.5% Triton-X 100 for 10 min. To block unspecific binding sites, the brains were incubated for 1 h at room temperature on a shaker in a 0.5% Triton-X 100 solution in PBS with 2% of normal goat serum (NGS, Jackson Immuno Research Laboratories). Subsequently, they were incubated on a shaker in primary antibody to synapsin from mouse 1 : 50 in PBS with 0.5% Triton-X 100 and 2% NGS for 5 days in a fridge at 4°C. The brains were then rinsed three times in PBS for 20 min and subsequently incubated in a secondary anti-mouse antibody from goat coupled to AlexaFluor 568 (A12380, Molecular Probes, Eugene, OR, USA) dye (1 : 250) and CF633 Phalloidin (2.5 µl Phalloidin from Methanol stock solution in 500 µl PBS) in PBS with 0.5% Triton-X 100 and 1% NGS for 3 days on a shaker at 4°C. After incubation, the brains were rinsed in PBS four times for 20 min each and post-fixed in 4% formaldehyde in PBS overnight at 4°C. They were washed four times in PBS for 20 min each and then dehydrated in an ascending ethanol series (30%, 50%, 70%, 90%, 95% for 3–4 min each, and two times in 100% ethanol for 5 min). The brains were cleared in methyl salicylate (4529.1, Carl Roth Gmbh & Co. KG, Karlsruhe, Germany) and finally transported from the field laboratory to the University of Würzburg for confocal laser scanning microscopy and further analyses.

(d) Quantification and statistical analysis

(i) Data analysis

A confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems GmbH, Wetzlar, Germany) was used for scanning the brains as image stacks at a step size of 5 µm. APO 20×/0.7 IMM water immersion objective with a step size of 5 µm for overviews with 0.75 digital zoom for whole-brain scans, with 1.6 zoom for close-ups of the CX, with 2.0 zoom for the mushroom body calyx and with 4.0 zoom for detailed scans of the lateral complex. A PL APO 63×/1.2 W objective with 2.0 digital zoom with a step size of 0.5 µm was used for high-resolution scans in the collar of the mushroom body calyx.

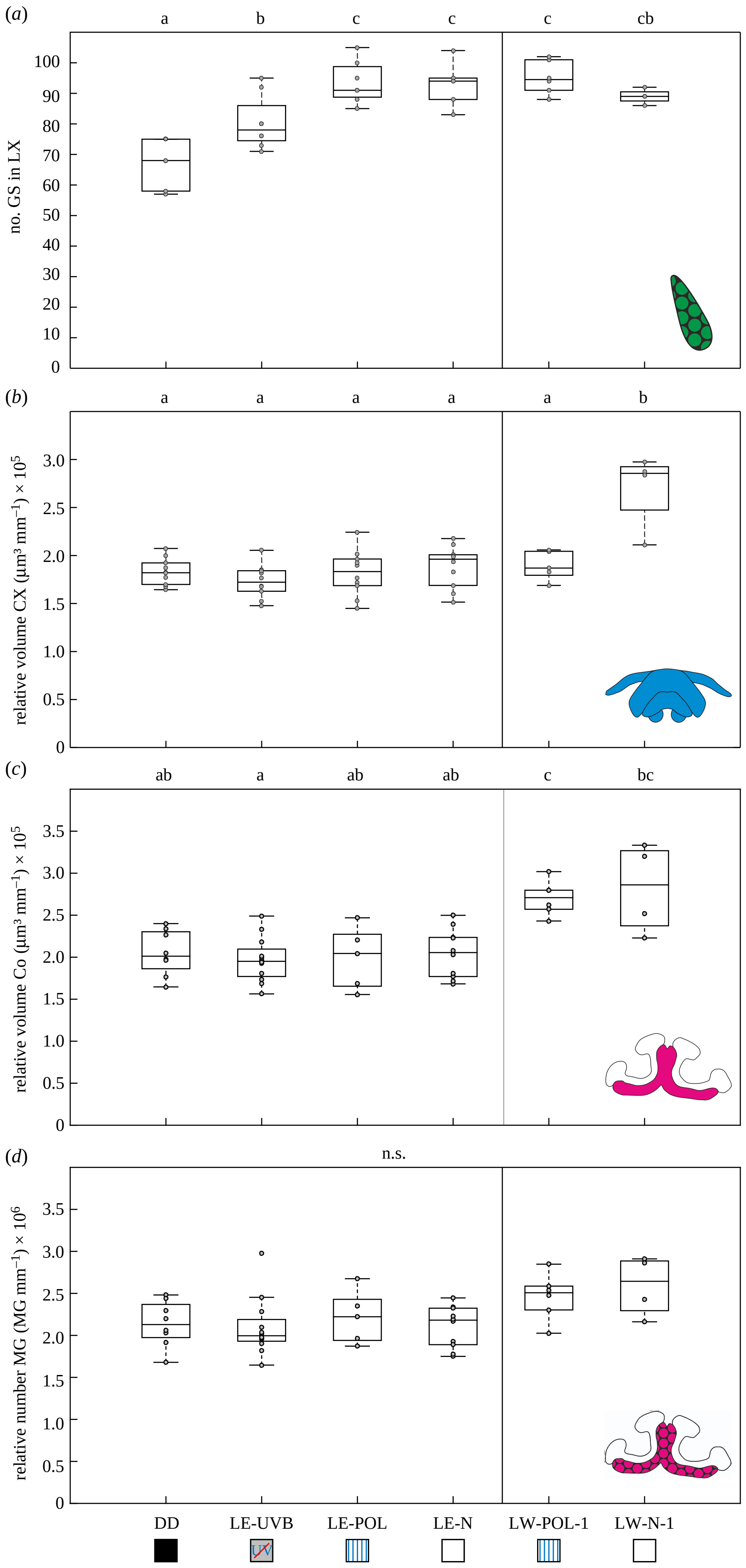

Figure 2. (*Caption opposite.*)

Figure 2. (*Opposite.*) The influence of LE and LW on neuronal plasticity. Neuronal changes after 3 days in complete darkness (DD) compared to LE under skylight with a UV cut-off filter (LE-UVB), under full-spectrum skylight with a linear polarization filter (LE-POL) and under natural skylight (LE-N) as well as after 3 days of LW under full-spectrum skylight with a linear polarization filter (LW-POL-1) and under natural skylight (LW-N-1). (*a*) The absolute number of synaptic complexes is shown for the giant synapses (GS; $n_{\mathrm{DD}} = 6$, $n_{\mathrm{LE\text{-}UVB}} = 8$, $n_{\mathrm{LE\text{-}POL}} = 7$, $n_{\mathrm{LE\text{-}N}} = 6$, $n_{\mathrm{LW\text{-}POL\text{-}1}} = 6$, $n_{\mathrm{LW\text{-}N\text{-}1}} = 4$) in the lateral complex (LX). The size-corrected volumes are shown for (*b*) the central complex (CX; $n_{\mathrm{DD}} = 10$, $n_{\mathrm{LE\text{-}UVB}} = 10$, $n_{\mathrm{LE\text{-}POL}} = 10$, $n_{\mathrm{LE\text{-}N}} = 10$, $n_{\mathrm{LW\text{-}POL\text{-}1}} = 5$, $n_{\mathrm{LW\text{-}N\text{-}1}} = 4$) and (*c*) the mushroom body collar (Co; $n_{\mathrm{DD}} = 8$, $n_{\mathrm{LE\text{-}UVB}} = 12$, $n_{\mathrm{LE\text{-}POL}} = 5$, $n_{\mathrm{LE\text{-}N}} = 10$, $n_{\mathrm{LW\text{-}POL\text{-}1}} = 6$, $n_{\mathrm{LW\text{-}N\text{-}1}} = 4$). (*d*) The size-corrected total number of MG is shown for the mushroom body collar ($n_{\mathrm{DD}} = 8$, $n_{\mathrm{LE\text{-}UVB}} = 12$, $n_{\mathrm{LE\text{-}POL}} = 5$, $n_{\mathrm{LE\text{-}N}} = 10$, $n_{\mathrm{LW\text{-}POL\text{-}1}} = 6$, $n_{\mathrm{LW\text{-}N\text{-}1}} = 4$). The central line of each boxplot depicts the median of the data. The upper and lower limits of the boxes show the 25th and 75th percentiles, while the whiskers extend to the extreme data points without outliers. Outliers are values that are more than 1.5 times the interquartile range from the edge of the box. All data points are plotted as grey circles. Significant differences between the groups are marked with lower case letters (Kruskal–Wallis test ($\alpha = 0.05$) and post hoc Mann–Whitney U-test with Benjamini–Hochberg correction (false discovery rate of $\alpha = 0.05$)). For statistical details, see text. (Online version in colour.)

The neuropils and their subunits can easily be distinguished in anti-synapsin labelled whole mount brains (figure 1*c*). The volumes of the CX, the mushroom body and their subunits were analysed using the three-dimensional reconstruction software TrakEM2 [20] plugin for ImageJ 1.52n (Wayne Rasband, National Institutes of Health, USA).

For analysing structural synaptic plasticity during LE and LW, microglomeruli (MG) were quantified with Amira (Amira-Avizo Software 2019.1, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) in the visual subregions of the mushroom body calyx (collar) using the protocol established by Groh *et al.* [21]. Anti-synapsin, phalloidin labelled synaptic boutons were counted in three defined volumes (1000 μm^3 each) by a person who was blind to the experimental treatments. MG density was calculated by averaging the analysed volumes of the collar as numbers of MG per 1000 μm^3. The total numbers of MG per collar were estimated by extrapolation of the average MG numbers per 1000 μm^3 to the total volume of the collar. This represents a good approximation, as MG densities are largely homogeneous throughout the collar of *C. nodus* neuropils [4,22]. Giant synapses in the lateral bulb of the lateral complex in one brain hemisphere of each experimental ant were traced and quantified using TrakEM2 [20] plugin for ImageJ 1.52n (Wayne Rasband, National Institutes of Health, USA) using the protocol established by Grob *et al.* [23].

(ii) Statistics

Since *C. nodus* ants' sizes and, therefore, brain volumes showed differences between the experimental groups, the size of the neuropils was corrected for the ants' sizes (which correlates with the ants' total brain size), by dividing the volume by the thorax length, which is an established measure for body size [24]. The neuroanatomical differences between the groups were compared using a Kruskal–Wallis test ($\alpha = 0.05$) and a post hoc Mann–Whitney U-test with Benjamini–Hochberg correction [25] (false discovery rate $\alpha = 0.05$). All descriptive statistical analyses were done with MATLAB (2015a or 2018a, The MathWorks Inc., Natick, MA, USA).

(e) Nomenclature

For the nomenclature of neuropils in the ant brain, we refer to Habenstein *et al.* [19] (see also https://www.insectbraindb.org for 3D data of the *Cataglyphis* brain) and Ito *et al.* [26]. For the terminology related to spatial orientation and navigation, we refer to Grob *et al.* [14].

3. Results and discussion

(a) Light exposure

Celestial compass information provided by the polarization pattern of the skylight, the most important navigation cue for *Cataglyphis* desert ants [1,27], is transferred from the dorsal rim area of the compound eyes and optic lobes to the CX via the anterior optic tract and the bulbs of the lateral complex [3,4,18]. In the lateral-complex bulbs, the last synaptic relay station of the sky-compass pathway before the CX, sensory exposure to manipulated skylight (LE) led to an increase in the number of large synaptic complexes (giant synapses) dependent on the light spectra perceived during first LE (figure 2*a*; Kruskal–Wallis test: number giant synapses: $\chi = 21.29$, $n = 37$, $p = 0.0007$; $n_{\mathrm{DD}} = 6$, $n_{\mathrm{LE\text{-}UVB}} = 8$, $n_{\mathrm{LE\text{-}POL}} = 7$, $n_{\mathrm{LE\text{-}N}} = 6$, $n_{\mathrm{LW\text{-}POL\text{-}1}} = 6$, $n_{\mathrm{LW\text{-}N\text{-}1}} = 4$). The increase was more prominent when the ants were exposed to the full light spectrum compared to skylight without UV-light or constant darkness (figure 2*a*; numbers giant synapses: median (minimum/maximum): DD = 68 (57/75), LE-UVB = 78 (71/95), LE-POL = 91 (85/105), LE-N = 94 (83/104), LW-POL-1 = 94,5 (88/102), LW-N-1 = 89 (86/92)). The light spectrum-dependent increase in bulb giant synapse numbers is comparable with the increase previously found under similar conditions in *Cataglyphis fortis* [18]. UV-light is an important navigation cue for desert ants, since they only perceive the polarization pattern of the sky in the UV-light spectrum [28]. Interestingly, the increase in the numbers of giant synapses occurred independently of a changing polarization pattern (figures 2*a* and 3*a*) and in the absence of LW behaviour (figure 2*a*). This was different in the CX, the target integration centre of the anterior optic tract downstream from the lateral-complex bulb synapses. Here, a volume increase was triggered only when the ants had been able to experience natural skylight with a changing polarization pattern during 3 days of performing initial LW (figure 2*b*, Kruskal–Wallis test: relative CX volume: $\chi = 14.09$, $n = 49$, $p = 0.0151$; $n_{\mathrm{DD}} = 10$, $n_{\mathrm{LE\text{-}UVB}} = 10$, $n_{\mathrm{LE\text{-}POL}} = 10$, $n_{\mathrm{LE\text{-}N}} = 10$, $n_{\mathrm{LW\text{-}POL\text{-}1}} = 5$, $n_{\mathrm{LW\text{-}N\text{-}1}} = 4$). Contrary to the lateral-complex bulb synapses, exposure to natural skylight alone was not sufficient to trigger volumetric changes in the CX (figure 2*b*). We hypothesize that structural plasticity in lateral-complex bulb synapses promotes stabilization of the sky-compass network in downstream CX circuits via inhibitory tangential neurons (ring neurons in *Drosophila*) to adapt the neuronal network to new sensory input (homeostatic plasticity) [3,18,29].

Visual information, e.g. about the panorama and the sky, is transferred to the mushroom bodies, mainly via the anterior superior optic tract [3,4,19]. Like in the CX, the volume of the visual input region of the mushroom body calyx (collar) did not increase when the ants were exposed to skylight in the absence of LW. Only when the ants were able to perform LW, the volumes of the mushroom body collar regions increased (figure 2*c*; Kruskal–Wallis test:

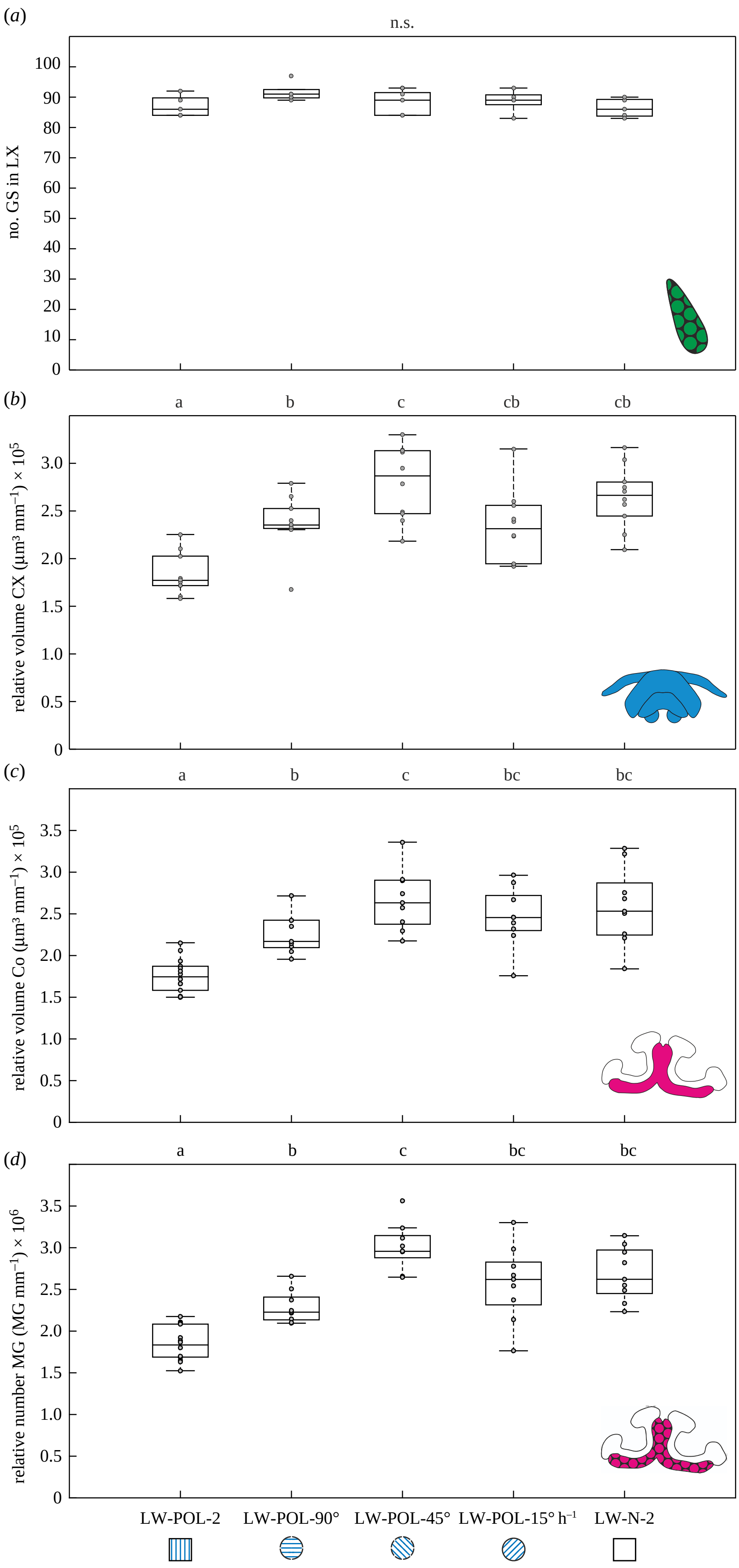

Figure 3. (*Caption opposite.*)

Figure 3. (*Opposite.*) The influence of a rotating polarization pattern on neuronal plasticity following LW. Neuronal changes after 3 days LW under different skylight conditions. The labels indicate LW together with the light filter conditions (either under a polarizer (POL) or under natural light conditions (N)): stationary linear polarization filter (LW-POL-2), linear polarization filter that was turned by 90° once per day at solar noon (LW-POL-90°), linear polarization filter that was turned hourly by 45° (LW-POL-45°), linear polarization filter that was turned constantly at a speed of $15^\circ\,h^{-1}$ (LW-POL-$15^\circ\,h^{-1}$) and the natural skylight (LW-N-2). (*a*) The absolute numbers of the synaptic complexes in the lateral complex (giant synapses, GS; $n_{LW\text{-}POL\text{-}2} = 5$, $n_{LW\text{-}POL\text{-}90^\circ} = 5$, $n_{LW\text{-}POL\text{-}45^\circ} = 5$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 5$, $n_{LW\text{-}N\text{-}2} = 5$) are shown. The size-corrected volumes are shown for (*b*) the central complex (CX; $n_{LW\text{-}POL\text{-}2} = 10$, $n_{LW\text{-}POL\text{-}90^\circ} = 10$, $n_{LW\text{-}POL\text{-}45^\circ} = 10$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 10$, $n_{LW\text{-}N\text{-}2} = 10$) and (*c*) the mushroom body collar (Co; $n_{LW\text{-}POL\text{-}2} = 14$, $n_{LW\text{-}POL\text{-}90^\circ} = 9$, $n_{LW\text{-}POL\text{-}45^\circ} = 9$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 9$, $n_{LW\text{-}N\text{-}2} = 9$). (*d*) The size-corrected total numbers of MG are shown for the collar ($n_{LW\text{-}POL\text{-}2} = 14$, $n_{LW\text{-}POL\text{-}90^\circ} = 9$, $n_{LW\text{-}POL\text{-}45^\circ} = 9$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 9$, $n_{LW\text{-}N\text{-}2} = 9$). For figure conventions, figure 2, and for statistical details, see text. (Online version in colour.)

relative mushroom body volume: $\chi = 20.11$, $n = 45$, $p = 0.0012$; $n_{DD} = 8$, $n_{LE\text{-}UVB} = 12$, $n_{LE\text{-}POL} = 5$, $n_{LE\text{-}N} = 10$, $n_{LW\text{-}POL\text{-}1} = 6$, $n_{LW\text{-}N\text{-}1} = 4$). The dependency on the early learning behaviour of desert ants suggests that volume-related structural neuronal plasticity in the collar is triggered by learning and long-term memory formation. However, we did not find changes in the relative numbers of synaptic complexes (MG) in the mushroom body calyx (figure 2*d*; Kruskal–Wallis test: 2D: relative number MG: $\chi = 10.48$, $n = 45$, $p = 0.0626$; $n_{DD} = 8$, $n_{LE\text{-}UVB} = 12$, $n_{LE\text{-}POL} = 5$, $n_{LE\text{-}N} = 10$, $n_{LW\text{-}POL\text{-}1} = 6$, $n_{LW\text{-}N\text{-}1} = 4$). This contrasts with previous results showing that both the numbers of MG and the volumes of the mushroom body collar increase after the performance of LW under natural skylight [4], which is also confirmed by our current experiments using a rotating polarization filter (see below and figure 3). We assume that this difference in mushroom body plasticity might be due to either colony-level variance or other biological variables. The mushroom bodies are essential for processing visual information about the landmark panorama [3,12,13]. Consequently, the quality of panoramic information may exert additional influences on LW-related plasticity in the mushroom bodies. While the LW experiments that revealed a significant influence of the skylight on mushroom body plasticity ([4], and figure 3) were performed in clearings of Schinias National Park, the experiment shown in figure 2*c*,*d* was performed in the more cluttered environment of Strofylia National Park. This provides an important hint for future experiments to investigate the influence of the panorama on mushroom body plasticity during LW in a more controlled situation. Interestingly, long-term LE (5 days) leads to pruning in MG numbers in *C. fortis* [10], while 3 days of LE in *C. nodus* (figure 2*c*,*d*) did not lead to a significant change in MG numbers. This indicates that LE-related synaptic pruning in the mushroom bodies obviously requires longer stimulus exposure compared to the exposure time required for LW-related increases of mushroom body synaptic complexes [4,6,30].

Overall, this means that homeostatic synaptic plasticity following longer LE occurs along with both visual pathways [3,29]. The majority of tangential neurons projecting from the lateral complex to the CX are GABAergic [18], whereas projection neurons providing input to the mushroom bodies are presumably cholinergic [31]. As a consequence, the increase in giant synapses in the lateral-complex bulb, in contrast with pruning of MG in the mushroom body, could be attributed to different functional properties of the input neurons [3]. Ants are exposed to skylight before they start to perform LW, e.g. while looking out of the nest entrance, or while leaving the nest for seconds during waste disposal or digging [6]. Here, visual pathways could already undergo homeostatic plasticity preparing the ant's brain for the drastic changes in their visual environment.

(b) Rotation of polarization pattern

Learning-dependent neuronal plasticity in the CX, and in previous experiments also in the mushroom bodies (figure 2 and Grob *et al.* [4]), occurred only when the ants were able to perform LW under natural skylight. It was absent when the polarization pattern was stationary. Therefore, using a linear polarizer (POL), we tested whether rotation of a polarization pattern over the day is a necessary change in skylight cues to trigger neuronal plasticity after LW in a natural habitat (figure 3). As expected from the result shown in figure 2*a*, rotating POL had no influence on the number of lateral-complex bulb giant synapses (figure 3*a*, Kruskal–Wallis test: number giant synapses: $\chi = 5.45$, $n = 25$, $p = 0.2438$; $n_{LW\text{-}POL\text{-}2} = 5$, $n_{LW\text{-}POL\text{-}90^\circ} = 5$, $n_{LW\text{-}POL\text{-}45^\circ} = 5$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 5$, $n_{LW\text{-}N\text{-}2} = 5$; number giant synapses: median(minimum/maximum): LW-POL-2 = 86 (84/92), LW-POL-90° = 91 (89/97), LW-POL-45° = 89 (84/93), LW-POL-$15^\circ\,h^{-1}$ = 89 (83/93), LW-N-2 = 86 (83/90)). This reaffirms that structural synaptic plasticity in the lateral-complex bulb is independent from both a changing polarization pattern and LW behaviour. By contrast, a single 90° rotation of POL at solar noon, as well as rotating the POL either by 45° every hour, or continuously at a speed of $15^\circ\ h^{-1}$ (figure 1*b*) led to a comparable increase in CX volume as under natural skylight (figure 3*b*; Kruskal–Wallis test: relative CX volume: $\chi = 25.23$, $n = 50$, $p < 0.0001$; $n_{LW\text{-}POL\text{-}2} = 10$, $n_{LW\text{-}POL\text{-}90^\circ} = 10$, $n_{LW\text{-}POL\text{-}45^\circ} = 10$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 10$, $n_{LW\text{-}N\text{-}2} = 10$).

Similar to the pattern of results observed in the CX, a single rotation of POL per day triggered a volume increase in the mushroom body collar (figure 3*c*; Kruskal–Wallis test: relative mushroom body volume: $\chi = 10.25$, $n = 50$, $p = 0.0363$; $n_{LW\text{-}POL\text{-}2} = 14$, $n_{LW\text{-}POL\text{-}90^\circ} = 9$, $n_{LW\text{-}POL\text{-}45^\circ} = 9$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 9$, $n_{LW\text{-}N\text{-}2} = 9$). Likewise, the number of MG in the collar increased depending on a rotating POL (figure 3*d*; Kruskal–Wallis test: relative number MG: $\chi = 34.27$, $n = 50$, $p < 0.0001$; $n_{LW\text{-}POL\text{-}2} = 14$, $n_{LW\text{-}POL\text{-}90^\circ} = 9$, $n_{LW\text{-}POL\text{-}45^\circ} = 9$, $n_{LW\text{-}POL\text{-}15^\circ\,h^{-1}} = 9$, $n_{LW\text{-}N\text{-}2} = 9$). The mushroom body plasticity after LW under a changing polarization pattern is in line with previous experiments [4]. The results demonstrate that the moving skylight polarization pattern during initial LW represents the necessary skylight cue for triggering learning-dependent changes in visual integration centres in the *Cataglyphis* brain associated with sky-compass processing. By contrast, the movement of the sun without a (moving) polarization pattern is not sufficient to trigger the LW-related neuronal changes in both visual integration centres. This hierarchy in cue importance is reflected in the orientation behaviour of experienced foragers. *Cataglyphis* foragers preferentially rely on directional information provided by the polarization pattern of the sky compared to the position of the sun [27]. While the direct position of the sun and the polarization pattern are detected in different parts of the eye [3], directional information from the two sources of information are processed by the same neurons in the anterior optic tubercle of the insect brain [32–35]. In the CX, directional information from the sun and the polarization pattern of the sky are integrated to complement each other to provide a robust celestial compass [15,33].

(c) Acquisition of the solar ephemeris

Our results support the hypothesis that *Cataglyphis* ants use the changing sky polarization pattern to learn the solar ephemeris during their initial LW outside the nest [3,4,6,7,36]. Desert ant foragers rely on a time-compensated sun compass during their long and far-ranging foraging runs, for which they have to learn and memorize the seasonal course of the sun over the day [2,5,36]. This is also crucial for repeated visits to the same food source over extended periods. The transition from dark-adapted interior worker to visually guided forager in bright skylight requires the calibration of the ants' visual guidance systems [3,29]. During their LW, *C. nodus* ants perform a specific turn besides pirouettes, so-called voltes, that may allow them to read out the sky polarization pattern [6]. This is supported by the fact that closely related *C. fortis*, living in almost featureless environments, exclusively perform voltes, but not pirouettes with nest-directed stops [6].

The neuronal mechanisms for the storage of an internal representation of the solar ephemeris during initial LW are still unknown. Computational models demonstrate that the CX circuitry is able to store spatial (heading) information [37] based on orientation cues from visual and other modalities [38]. This makes the CX a suitable candidate for a memory-based calibration of visual compass cues, which is also supported by the learning-dependent volume increase of the CX shown in our present study. The differences in the synaptic architecture and behavioural function of the CX and mushroom body visual pathways inferred from circuit analyses and ablation experiments suggest that view-based panoramic information is stored in the mushroom bodies, whereas directional information is processed in the CX [12,13,29,39,40]. It is not possible with the methods currently available to quantify distinct synapses or pre- and postsynaptic elements within subunits of the CX (as it is possible for synaptic MG in the mushroom bodies) for exploring the exact source of volume increase. This could potentially be due to an increase in the number of synaptic connections, but also to the extension of dendrites as shown in the mushroom bodies [29]. The CX is involved in several tasks related to spatial orientation like the directional control of movement [41,42], sun orientation [11], landmark orientation [43] and angular path integration [43]. The CX, therefore, is an important integration centre for multisensory orientation cues [44] making it a well-suited neuropil to combine polarization information with other directional cues, for example, terrestrial reference systems.

Due to the large numbers of Kenyon cells, the mushroom bodies in *Cataglyphis* brains are well-equipped to store many celestial snapshots. Many thousands of microcircuits in the mushroom body collar provide a suitable neuronal substrate for this [4,16]. Such snapshot-based learning mechanisms have been extensively studied in view-based learning of the visual panorama during hymenopteran LW and flights [45–47]. Furthermore, UV light is important for determining directions based on the visual panorama [48]. Therefore, UV light is crucial for different view-based navigational strategies, but it remains unclear whether polarized light information is also transferred to the mushroom bodies. Our results demonstrate that LW-related neuroplasticity in the mushroom bodies is dependent on polarization information suggesting that information about a changing polarization pattern is somehow registered in the mushroom bodies. This might be achieved via direct transfer by polarization-sensitive neurons or indirect feedback via modulatory information from other brain areas, which needs to be addressed in future physiological studies. Finally, for sky-compass calibration, the information about sky polarization dynamics needs to be combined with input from the endogenous clock, which also requires future investigation to fully understand the processes underlying the acquisition of an internal ephemeris [3].

In order to learn the rotation of the sky, animals need a geostable reference [2,5,36]. Experienced honeybees use the landmark panorama for re-calibrating the movement of celestial compass cues [49,50]. Since naive *C. nodus* use a magnetic compass during initial LW [8], our hypothesis is that the earth's magnetic field provides the geostable reference system with which the solar ephemeris is initially calibrated [3,8,51]. This would allow the ants to calibrate the celestial compass and to learn the landmark panorama simultaneously during initial LW. As we propose for *Cataglyphis*, migratory birds are able to calibrate their celestial compass based on magnetic compass information [52]. Birds use a wide range of different navigation cues for their long-distance migration and adapt the cue hierarchy according to their navigational needs [53]. Similarly, *Cataglyphis* ants switch between different navigation cues and value them differently throughout their foraging careers [1,54,55,56]. The brain of *C. nodus* contains multimodal integration centres [19,57] that allow for the convergence of orientation cues from different sensory modalities. To become a successful forager, compass information from the polarization pattern of the sky needs to be further integrated with input from the magnetic compass, sun position and internal clock [3]. The present results suggest that early experience of a moving polarization pattern together with a geostable magnetosensory input [8] during initial LW in the natural habitat provide the crucial multimodal sensory input necessary to trigger neuroplastic changes resulting in calibrations of the visual compass systems.

Data accessibility. All processed data are supplied within the manuscript and electronic supplementary material [58]. Raw data (confocal image stacks of whole brains and 3D data) will be made available from the authors upon request.

Authors' contributions. R.G.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization and writing—original draft; O.H.C.: formal analysis and investigation; K.P.: conceptualization and writing—review and editing; K.G.: formal analysis and methodology; W.R.: conceptualization, funding acquisition, project administration, supervision and writing—review and editing; P.F.: conceptualization, funding acquisition, investigation, project administration, supervision and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. The authors declare no competing interests.

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7 General Discussion

7.1 A Brain Suited for Navigation

Desert ants of the genus *Cataglyphis* are expert navigators. These solitary foragers rely heavily on visual guided navigation (Wehner, 2020). While foraging during the hottest times of the day, the soil temperature may reach life-threatening 70°C. Such foraging trips may exceed 1,500 m, equivalent to several thousand times the ants' body lengths (Buehlmann et al., 2014; Grah and Ronacher, 2008; Huber and Knaden, 2015; Knaden and Graham, 2016; Wehner, 2020). Still, the ants are able to find the most direct and shortest route back to their safe nest. Under such high selection pressure, *Cataglyphis* has evolved an extraordinary neuronal basis for their vast set of navigational skills.

7.1.1 Neuronal Adaptations to Different Navigational Needs

Desert ants are best studied for their navigational capabilities during foraging. However, there is a caste with a very different task, namely reproduction. While the worker caste only includes female ants, the reproductive caste comprises both, male and female ants (Wilson and Hölldobler, 2005). In ants of the genus *Cataglyphis*, the social structure and how division of reproduction is manifested is very diverse. Depending on the species, *Cataglyphis* colonies can have just a single or multiple reproductive queens. These queens can be either singly- or multiply-mated. In some cases, the workers are also able to produce offspring (Boulay et al., 2017). This diversity in social and mating structures is an adaptation to the ants' harsh habitat. While all desert ants are marvellous navigators with similar foraging traits, the diversity of social, breeding, and dispersal strategies within the genus *Cataglyphis* enabled them to populate a vast distributional range of desert habitats with many species coexisting without competition (Boulay et al., 2017; Wehner, 2020). Thus, *Cataglyphis* ants possess a wide range of mating (Leniaud et al., 2011) and dispersal strategies (Peeters and Aron, 2017).

These strategies provide the ants with very specific navigational needs, which are reflected in the ants' behavioral and neuronal adaptations. In contrast to some *Cataglyphis* species, both sexes of the reproductive castes in *Cataglyphis nodus* are winged and fly out of their nest of birth. This airborne orientation is reflected in a slightly higher amount of sensory cells of the Johnston's organ of males and queens compared to workers (Chapter 3: Grob et al., 2021c). Since both sexes of the reproductive cast are capable of flight, *C. nodus* might use a 'mating swarm' (also called 'male aggregation') mating behaviors, where reproductive animals of several colonies meet away from their natal nest to mate (Peeters and Aron, 2017). Males and virgin queens (gynes) are both faced with the crucial challenge to orient themselves and find suitable mating partners. Afterwards, mated queens must find a suitable spot to found a new colony on their own.

These orientational challenges ask for innate (in males) and learned behavioral spatial orientation responses (in females). These evolutionary needs for orientation are reflected in the size and structure of the associated brain neuropils of ants (Gronenberg, 2008): Males use non-compass orientation based in pheromone communication and visual cues to detect gynes. This is reflected in their neuronal architecture. Male ants have massively enlarged glomeruli in the antennal lobes (*A. vollenweideri* (Kuebler et al., 2010), *C. fortis* (Stieb et al., 2011), *C. nodus* (Chapter 5: Grob et al., 2021b), *C. japonicus* (Nishikawa et al., 2008), *H. saltator* (Hoyer et al., 2005)).

In contrast, female ants, especially the worker caste, have to repeatably find their way back to the same spot, i.e. their nest or a profitable food patch. They rely heavily on orientation behaviors based on learned information, like the visual panorama, or different types of landmark cues (Fleischmann et al., 2016, 2018b; Huber and Knaden, 2017, 2018; Steck et al., 2009; Wehner, 2020; Wehner et al., 1996). To do so, they need a brain that is adapted to store a vast amount of learned orientation information. The mushroom bodies are essential neuropils for memory- and view-based orientation (Buehlmann et al., 2020; Kamhi et al., 2020). Thus, the amount of synaptic complexes in the mushroom bodies is especially high in female *C. nodus* (gynes and workers (☿)) and smaller in males both in the visual (collar) (Table 1) and in the olfactory (lip) (Table 2) input regions (Chapter 5: Grob et al., 2021b). The microcircuits within the mushroom bodies show high synaptic plasticity throughout the ontogeny of an ant worker lifetime (Table 1 & 2). This will be discussed in more detail later.

Table 1: Absolute Numbers of Microglomeruli in Mushroom Body Collars*

	Males	Gynes	Interior ☿	Novice ☿	Forager ☿
Median	195,543	397,614	359,139	434,014	397,359
Median absolute deviation (±)	2,090	2,760	3,244	2,849	4,003
n	4	5	14	13	8

Table 2:Absolute Number of Microglomeruli in Mushroom Body Lips*

	Males	Gynes	Interior ☿	Novice ☿	Forager ☿
Median	282,692	75,1819	688,659	779,375	695,431
Median absolute deviation (±)	26,619	51,511	49,870	68,469	76,710
n	4	5	5	13	8

*Data for males, gynes and foragers based on Grob et al. (2021b), for interior workers and novices that had performed three days of learning walks under a natural sky based on (Grob et al., 2017, 2022).

The most important orientation cues for *Cataglyphis* worker during foraging are visual cues (Wehner, 2020). These include view-based visual orientation cues (local cues, non-compass orientation (Grob et al., 2021a)) from landmarks, and the panorama (Collett, 2010; Collett et al., 1992; Fleischmann et al., 2016, 2018b; Ronacher and Wehner, 1995; Wehner et al., 1996; Ziegler and Wehner, 1997) and celestial compass cues (global cues, compass orientation (Grob et al., 2021a)) based mainly on based on the sun's position and the polarization pattern of the sky (Wehner, 1989; Wehner and Müller, 2006) (Figure 4). Two neuropils have been shown to be essential for these two types of orientation behavior. The mushroom bodies, high-order sensory integration centers involved in learning and memory formation, have the capacity to store a large number of panoramic snapshots (Ardin et al., 2016) and have been shown to be involved in view-based orientation (Buehlmann et al., 2020; Kamhi et al., 2020). Compass orientation from the celestial compass is integrated into the central complex (Honkanen et al., 2019; Steinbeck et al., 2020; Stone et al., 2017). The central complex plays an essential role in ant orientation (Dell-Cronin et al., 2021). In the central complex polarization-vision signals are transferred from the upper unit of the central body to the contralateral hemisphere of the protocerebral bridge. The electric field vectors of polarized light are mapped as a topographic representation (Heinze and Homberg, 2007; Heinze and Reppert, 2012; Homberg et al., 2011). This map-like representation is the necessary bases for the use of the skylight compass (Honkanen et al., 2019; Pfeiffer and Homberg, 2014; Stone et al., 2017). The central complex and the mushroom bodies are prominent in the *Cataglyphis* brain (Habenstein et al., 2020). In the following, I will discuss the role of these neuropiles in the processing of directional information, both view-based (mushroom bodies) and compass information (central complex) in more detail (Figure 4).

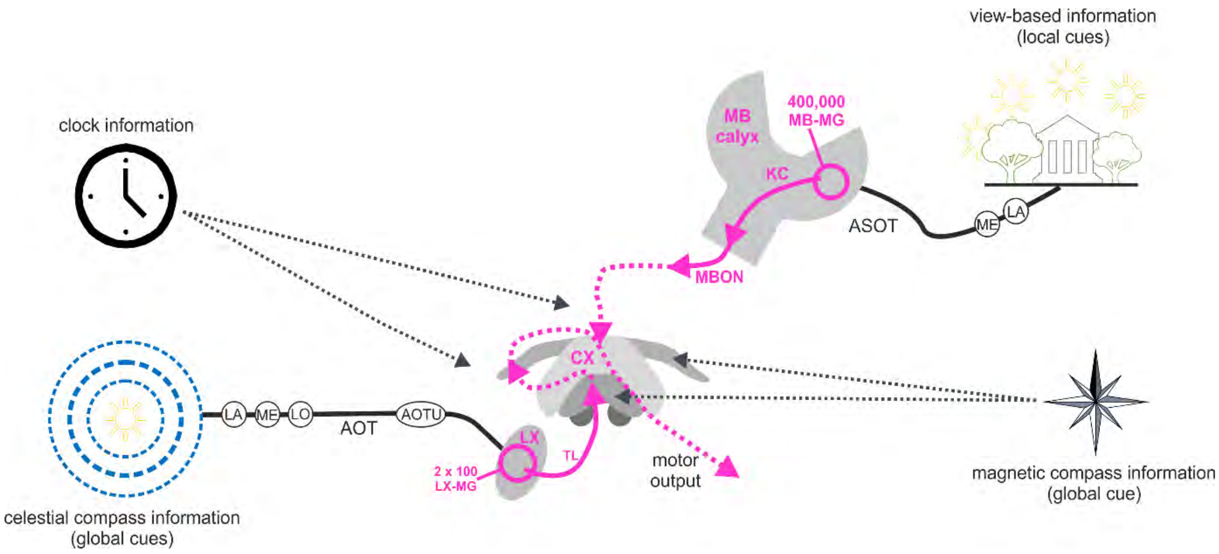

Figure 4 Model for processing of navigational information. View-based non-compass information (local cues), like panoramic information, is transferred via the anterior superior optic tract (ASOT) to the mushroom bodies (MB). Sky-compass information (global cues), in contrast, is transferred via the anterior optic tract (AOT) to the lateral (LX) and central complex (CX). The mushroom body output neurons (MBON) receive input from about 400,000 synaptic complexes (microglomeruli, MG) of the collar. A putative connection from the MBON into the CX and connections to the motor output are still hypothetical and depicted as dashed lines. The CX receives input from the ATO via about 100 MG (also called giant synapses) on each side. How sensory information for the geomagnetic compass and information from the endogenous clock for time-compensation are integrated is unknown. Further abbreviations: AOTU anterior optic tubercle, KC Kenyon cell, LA lamina, LO lobula, ME medulla, TL tangential neuron. (Figure adapted from Grob et al., 2019).

7.1.2 Processing of View-based Information in the Mushroom Bodies

Visual information is transmitted into high-order integrations centers in the *Cataglyphis* brain via two main visual tracts (Chapter 2: Grob et al., 2017; Habenstein et al., 2020). Behavioral studies in *C. fortis* had already predicted two separate visual tracts: one for global directional information and a second one for local memories (Müller, 1984). The central complex and the mushroom bodies have been suggested to be the neuronal substrate for path integration and orientation based on panoramic cues in *Cataglyphis*, respectively (Cruse and Wehner, 2011; Hoinville and Wehner, 2018; Stone et al., 2017; Webb and Wystrach, 2016). Additionally, behavioral experiments have shown that the mushroom bodies play a crucial role in orientation guided by learned landmarks (Buehlmann et al., 2020; Kamhi et al., 2020).

We described two major visual tracts that fit these premises (Chapter 2: Grob et al., 2017): The anterior superior optic tract directly transfers visual information about the panoramic scenery from the medulla to the ipsilateral and contralateral mushroom body collars (Figure 4) (Chapter 2: Grob et al., 2017, 2019; Rössler, 2019). There panoramic snapshots (local memories) could be stored in the densely packed synaptic network (Ardin et al., 2015; Grob et al., 2019). The presumably cholinergic synaptic complexes in the mushroom body collar (microglomeruli) (Bicker and Kreissl, 1994) undergo massive neuronal plastic changes during the transition phase from interior worker to outdoor forager. This is when the ants have to learn the visual panorama and landmark cues for the first time (Chapter 2: Grob et al., 2017; Chapter 6: Grob et al., 2022; Stieb et al., 2012). Before the ants start to perform learning walks, they are already getting exposed to the skylight, e.g., while looking out of the nest entrance or leaving the nest briefly during waste disposal or digging (Fleischmann et al., 2017; Zeil and Fleischmann, 2019). After first exposure to the natural skylight for five days, the number of synaptic complexes decreases (Stieb et al., 2012). This pruning of synaptic complexes might prepare the neuronal network for the drastic changes in sensory input, i.e., the switch from the dark nest to the bright outside world.

In contrast to this homeostatic plasticity, the mushroom body collar undergoes learning-related (Hebbian) plasticity when the ants perform learning walks. When the ants perform their learning walks either under the naturally changing skylight (Chapter 2: Grob et al., 2017) or under an artificially changing polarization pattern (Chapter 6: Grob et al., 2022) the number of microglomeruli in the mushroom body collar increases. After the transition phase, when the novices have become successful foragers, the number of microglomeruli in the mushroom bodies seems to decrease again (Table 1 & 2). Such pruning has been shown for *Cataglyphis* ants using electron and confocal microscopy techniques of postsynaptic structures (Seid and Wehner, 2009; Stieb et al., 2010). Similarly, the number of microglomeruli in the lip of *Acromyrmex ambiguous* increased within two days after olfactory avoidance learning followed by subsequent pruning (Falibene et al., 2015). An increase in synaptic complexes, i.e., the formation of new synapses, potentially is a suitable substrate for associative memory storage (Lamprecht and LeDoux, 2004; Moser, 1999). However, if long-term memory formation would always need new synaptic connections, memory capacity would likely be retained simply by the available space. It has been proposed that while new synaptic connections are formed for long-time memory formation, older, less used connections are eliminated after learning. This would result in the reorganization of the associative network while keeping the number of synaptic connections relatively stable (Falibene et al., 2015).

Like view-based panoramic orientation, it has also been suggested that sun orientation might be based on celestial snapshots (el Jundi et al., 2016). The mushroom bodies of *Cataglyphis* are well-equipped to store many celestial and panoramic snapshots. The many microcircuits formed by projection neurons and Kenyon cells in the mushroom-body collar provide a suitable neuronal substrate for this (Ardin et al., 2016; Grob et al., 2017). Such snapshot-based learning mechanisms have been studied more extensively in visual panorama learning, especially during hymenopteran learning walks and flights (Cartwright and Collett, 1983; Collett, 2010; Zeil, 2012). The role of the mushroom bodies for orientation based on learned landmark cues is also reflected in their size. *Cataglyphis* possess especially large mushroom bodies (Habenstein et al., 2020). In *C. nodus*, females (both workers and gynes), who live longer and need to be better adapted for storing memories than males, have larger mushroom bodies than males (Chapter 5: Grob et al., 2021b).

It is not yet known how and if polarization information is projected into the mushroom bodies in *Cataglyphis*. However, learning-walk related neuroplasticity in the mushroom bodies is dependent on polarization information (Chapter 6: Grob et al., 2022). This suggest that information about changes in the polarization pattern is registered in the mushroom bodies. Polarization information could enter the mushroom bodies either via direct transfer by polarization-sensitive projection neurons from the medulla or indirect via modulatory information from other brain areas. The integration of polarization information into the mushroom bodies needs to be addressed in future physiological studies.

7.1.3 Processing of Compass Information in the Central Complex

Global directional information from the ant's celestial compass is transferred via the anterior optic tract to the anterior optic tubercle (Figure 4) (Chapter 2: Grob et al., 2017; Schmitt et al., 2016). In the lateral complex bulb, the information is relayed via large synaptic complexes (giant synapses) to GABAergic tangential neurons that project into the lower unit of the central complex (Schmitt et al., 2016). The number of these synapses increases during first light exposure depending on the light spectra, especially UV-light (Chapter 6: Grob et al., 2022; Schmitt et al., 2016). This increase in the number of synaptic complexes in the lateral complex bulb is in sharp contrast to the decrease of microglomeruli in the mushroom body collar after first light exposure (Stieb et al., 2012). However, as mentioned above, tangential neurons projecting from the lateral bulbs to the central complex are GABAergic (Schmitt et al., 2016), while projection neurons to the mushroom bodies are cholinergic (Bicker and Kreissl, 1994). The homeostatic plasticity of these functionally different input neurons could potentially lead to the same outcome – the preparation of the neuronal network to a new sensory environment (Grob et al., 2019). Pruning of cholinergic synaptic connections in the mushroom bodies would lead to a less sensitive neuronal network. Likewise, increasing the amount of GABAergic synaptic connections in the lateral complex bulbs would also lead to a less sensitive neuronal network. Thus, despite neuronal changes in different directions, the homeostatic plasticity in the synaptic network of the mushroom bodies and the later complex bulbs would adapt the network for the high amount of sensory stimuli in the bright environment outside of the dark nest.

Once outside of the nest, UV-light plays an important role in the ants' celestial compass. *Cataglyphis* perceives the polarization pattern of the skylight only in the UV spectrum (Duelli and Wehner, 1973). The anterior optic tract terminates in the anterior optic tubercle and polarization information is projected from there to the central complex (Chapter 2: Grob et al., 2017; Habenstein et al., 2020; Homberg et al., 2011; Schmitt et al., 2016). The central complex has been proposed to be the neuronal substrate for path integration and plays an important role in integrating orientation information (Heinze and Homberg, 2007, 2008; Honkanen et al., 2019; Pfeiffer and Homberg, 2014; Webb and Wystrach, 2016). It is involved in innate orientation (Dell-Cronin et al., 2021) and is in relative size larger in males compared to female *C. nodus* (Chapter 5: Grob et al., 2021b). The central complex undergoes massive neuronal changes during learning walks. These changes are dependent on a polarization pattern that changes throughout the day (Chapter 2: Grob et al., 2017, Chapter 6: Grob et al., 2022). The central complex circuitry is able to store spatial information (Fiore et al., 2017) based on visual and other orientation cues (Ofstad et al., 2011). While exposure to light is enough to trigger a volume increase in the central complex in *Drosophila* (Barth and Heisenberg, 1997), *Cataglyphis* novices need to be able to perform learning walks (Chapter 2: Grob et al., 2017; Chapter 6: Grob et al., 2022). This suggests that the plasticity found in the central complex is learning-dependent (Hebbian plasticity). Thus, the calibration of the celestial compass by learning the movement of the sun in the sky (solar ephemeris) might take place in the central complex. Unfortunately, it is not possible with the methods used in our studies to analyze the causes for the volume increase in the central complex in more detail. The volume increase could be due to an increase in the number of synaptic connections or to the extension of dendrites (Rössler, 2019). Future studies will have to focus on the neuronal mechanisms underlying the learning-dependent volume increase of *Cataglyphis*. Studies at the ultrastructural level (electron microscopy) or expansion microscopy, might allow for quantifying the synapses or even pre- and postsynaptic elements within the central complex like it is possible for microglomeruli in the mushroom bodies.

In the central complex, information about the current heading has to be integrated with other orientational information. For path integration, information about the heading direction has to be combined with distance information, e.g., from optic flow information or a stride integrator (Srinivasan, 2015). Speed information based on optic flow enters the central complex via the noduli and converges with directional information in the central complex (Stone et al., 2017). Distance and direction information must be continuously integrated and stored to update the path integrator (Wehner, 2003). The distance memory of path integration can be disrupted by cold anesthetizing foraging ants, while the overall directional information remains unaltered (Pisokas et al., 2022). This suggests that directional and distance information are maintained in a cartesian coordinate system.

In addition to directional information based on a celestial compass, the central complex also integrates other directional information (Honkanen et al., 2019). For orientation based on panoramic cues, visual memory from the mushroom bodies and path-integration information must be combined in the central complex. The mushroom-bodies play an essential role in orientation based on learned visual cues (Buehlmann et al., 2020; Kamhi et al., 2020). Until now, no connection between the mushroom body output neurons and the central complex has been found in *Cataglyphis* (Grob et al., 2019; Rössler, 2019). However, recent connectome studies in *Drosophila* found direct connections from mushroom body output neurons to the upper unit of the central body (in *Drosophila* called “fan-shaped body”) (Hulse et al., 2021; Scheffer et al., 2020). These connections could transfer view-based navigational information from the mushroom bodies to the path integration center in the central complex. Future studies should investigate the neuronal connections in *Cataglyphis* that project from the mushroom bodies to the central complex. It is likely, that such connection might be direct connections from the mushroom body output neurons, as it is the case in *Drosophila* (Hulse et al., 2021; Scheffer et al., 2020) or indirect via synaptic relay stations in other neuropils.

In order to calibrate the celestial compass, information about the polarization pattern of the sun and magnetic compass information (Chapter 3: Fleischmann et al., 2018a) has to be matched and coupled to the internal clock of the animal (Figure 4) (Grob et al., 2019). In monarch butterflies, the internal clock relevant for time-compensated orientation is located in the antennae (Merlin et al., 2009). There it is co-localized with the magnetic compass of the butterfly (Guerra et al., 2014). This co-localization of magnetic compass and internal clock in the antennae might aid calibration of the celestial compass. Non-olfactory information from the antennae of *C. nodus* is projected to the antennal mechanosensory and motor center. Some projections terminate more posterior in the posterior slope, the ventral complex, and the ventrolateral protocerebrum (Chapter 4: Grob et al., 2021c). In these neuropils, non-olfactory afferents of the ants’ antennae terminate in close proximity with visual information from afferent projections from the optic lobes (Habenstein et al., 2020; Ibbotson and Goodman, 1990; Maronde, 1991) and from the polarization-sensitive ocelli (Chapter 4: Grob et al., 2021c). The convergences of information from the antennae (e.g., wind compass and gravitation information, and possibly magnetic compass information) with visual information from the celestial compass and the panorama would allow these neuropils to be a suitable candidate to synchronize or even calibrate these orientation systems.

7.2 Learning to Navigate: The Birds and The Bees and The Ants

To become a successful navigator, animals must first learn local orientation cues and calibrate their compass systems. *Cataglyphis* desert ants learn the visual landmark panorama of their nest surroundings during their first excursions (Fleischmann et al., 2016, 2018b; Wehner et al., 2004). During these learning walks, ants that inhabit environments with a prominent landmark panorama perform pirouettes (Fleischmann et al., 2017; Wehner et al., 2004). During these turns, the ants gaze back to the nest entrance, possibly to take snapshots of the homewards direction (Cartwright and Collett, 1983; Fleischmann et al., 2017; Graham et al., 2010; Zeil, 2012; Zeil and Fleischmann, 2019). Panoramic snapshots could be stored in the large number of synaptic connections in the mushroom bodies (Ardin et al., 2015; Grob et al., 2019). *C. nodus* uses the geomagnetic field to align their gazes and possibly use it as the reference system to learn the position of the landmarks (Chapter 3: Fleischmann et al., 2018a). When honey bees learn the position of a feeder, they also use the magnetic field as a reference (Collett and Baron, 1994). However, the bees only used magnetic information when other orientation cues were unavailable (Collett and Baron, 1994), whereas *C. nodus* uses the magnetic field during their learning walks as the primary directional cue (Chapter 3: Fleischmann et al., 2018a). Both experiments indicate that the magnetic field is used to learn and memorize landmark information.

Honey bees also use the panorama as a geostable reference system. They are able to re-calibrate their celestial compass in relation to the panorama (Towne, 2008; Towne and Moscrip, 2008). However, since *Cataglyphis* cannot use the panorama for orientation before performing learning walks (Fleischmann, 2018; Fleischmann et al., 2016), it is an unlikely candidate for the reference to calibrate the celestial compass on, at least initially. In birds, magnetic information and possibly the magnetic compass plays an essential role in the development of celestial orientation (Weindler et al., 1996). We could show that naïve *Cataglyphis* do not use their celestial compass for path integration during initial learning walks (Chapter 2: Grob et al., 2017) but instead use a magnetic compass (Chapter 3: Fleischmann et al., 2018a). This suggests that magnetic information plays a crucial role in learning the panorama but might also provide the necessary reference to calibrate the celestial compass. While pirouettes during learning walks might promote learning of the landmark panorama, voltes might be used to calibrate the celestial compass.

In contrast to pirouettes, voltes are performed even by desert ants that inhabit featureless habitats that lack a prominent panorama (Fleischmann et al., 2017). Similar to the voltes, dung beetles perform 360°-rotations on top of their dung balls to take snapshots of skylight cues (el Jundi et al., 2016). Desert ants might use such celestial snapshots of the rotating skylight cues to systematically calibrate their celestial compass over the course of the day (Grob et al., 2019). We could show that the rotation of the polarization pattern is necessary to trigger learning-related neuroplasticity in neuropils related to learning and memory, and navigation. However, neuroplasticity is independent from the position of the sun and its own movement in the sky (Chapter 6: Grob et al., 2022). Our results suggest that early experience of sky-polarization dynamics is essential for neuronal sky-compass calibration (Chapter 2: Grob et al., 2017, Chapter 6: Grob et al., 2022).

Future experiments should investigate the role of the magnetic field in neuronal changes during learning walks. For example, ants could be presented during learning walks with either an eliminated horizontal component of the magnetic field, providing the ants with magnetic information without directional information, or a disarrayed magnetic field, similar to the spiral experiment presented in chapter 3 (Fleischmann et al., 2018a). A magnetic field that holds no directional information could have a similar effect on the neuroplasticity during learning walks, like eliminating or disturbing the polarization pattern of the skylight presented in chapter 2 (Grob et al., 2017). Likewise, a rotating magnetic field that follows the path of the sun could be presented to the ants. This would prevent the sun from moving relatively to the magnetic cues. Similar experiments had been started in relation to the panorama as a potential reference by using a rotatable channel that could follow the path of the sun (Fleischmann, 2018). Unfortunately, the crucial experiments with the rotating channel could not be realized.

The rotating channel and the rotating magnetic field would provide additional insight into the flexibility of the cue dominance in *Cataglyphis* navigation. During the first trips outside of their nest, *C. nodus* mainly relies on path integration using a magnetic compass (Fleischmann et al., 2018a, 2020b). However, experienced foragers almost exclusively use path integration based on their celestial compass during their far-ranging foraging runs. Only close to their nest, they switch their cue hierarchy in favor of local orientation cues, like the panorama (Wehner, 2020).

When and why the ants switch from the magnetic compass to the celestial compass remains an open question (Fleischmann et al., 2020b). Similar switches between magnetic and celestial compass in cue hierarchy have been observed in birds (Pakhomov and Chernetsov, 2020).

An interesting time point in *Cataglyphis* foraging life is when experienced foragers are forced to re-calibrate or re-learn their navigational strategies. There are two described changes in the environment that trigger the performance of learning walks (either initial- or re-learning). Changes in the ants' visual panorama, e.g., by installing an artificial landmark, forces experienced foragers to perform learning walks to re-learn the altered surroundings (Fleischmann et al., 2016, 2021; Müller, 1984; Müller and Wehner, 2010). Likewise, experimentally induced unexpected changes in the polarization pattern, like a sudden rotation of a linear polarizer, leads to an increase in both the number of initial learning walks performed by novices and the number of re-learning walks performed by already experienced foragers (Grob, 2016; Chapter 2: Grob et al., 2017). These re-learning walks differ from initial learning walks performed by novices. During re-learning walks, foragers circle the nest entrance before leaving the nest area to search for food. Novices, in contrast, walk small loops and return the nest after a learning walk. During pirouettes in re-learning walks, experienced foragers do not gaze back to the nest entrance during the longest stopping phase (Fleischmann et al., 2021).

Interestingly, experienced foragers also react differently to a manipulated magnetic field. While the gaze directions of novices during the longest stopping phases of pirouettes are changed predictably by rotating the horizontal component of the magnetic field (Fleischmann et al., 2018a), foragers do not use the magnetic field to align their gazes (Fleischmann et al., 2021). Nevertheless, magnetic manipulations during re-learning walks still lead to changes in gaze directions, indicating that foragers are still magneto-sensitive (Fleischmann et al., 2021).

Such a difference in the reaction to magnetic manipulations was also found in pigeons. Young pigeons strongly observe the relation between magnetic and celestial cues, while experienced ones ignore magnetic manipulations and rely solely on their already established celestial compass (Wiltschko and Wiltschko, 1990). In contrast to ants, birds seem not to perform a specific behavior to calibrate their compass systems. However, early exploratory flights promote faster calibration (Wiltschko and Wiltschko, 1981). Recent experiments have suggested that body alignment to the magnetic field during dawn and dusk might play an important role in compass calibration during bird migration (Bianco et al., 2022).

The well-defined learning-walk behavior of ants provides a suitable target for studying the flexibility of cue hierarchy throughout the lifetime of an animal. Future studies should focus on the relationship between celestial and magnetic compass, as well as panoramic information during initial and re-learning walks. Such behavioral studies will provide further information about the mechanisms underlying navigational flexibility.

7.3 The Characteristics of the Magnetic Compass in Desert Ants

Orientation based on magnetic cues has been extensively studied in animals that travel over hundreds or even thousands of kilometers (Dreyer et al., 2018; Lohmann, 2018; Lohmann and Lohmann, 2019; Reppert et al., 2010; Warrant et al., 2016; Wiltschko and Wiltschko, 1972, 2005). The geomagnetic field can be a reliable guide during these wide-range journeys and even provide a map-like coordinate system (Lohmann et al., 2007).

However, it has been suggested that a magnetic compass for short-distance navigation might be useful under the right circumstances (Wyeth, 2010). The learning walks of *Cataglyphis* are a prime example of short-distance navigation based on magnetic cues (Chapter 3: Fleischmann et al., 2018a). They meet every assumption made in Wyeth's hypotheses: First, the primary cue for orientation, i.e., *Cataglyphis'* celestial compass, is not yet feasible. Naïve ants have to perform learning walks first in order to calibrate their celestial compass systems. Second, their goal, i.e., nest entrance, is not moving. Third, the ants do not drift during their learning walks. Fourth, other spatial representations of the environment are not yet available and, therefore, constrained. The latter is also true since *Cataglyphis* needs to perform learning was first in order to learn the visual landmark panorama (Fleischmann et al., 2020b; Grob, 2016; Wyeth, 2010).

There are currently two main hypotheses on how the geomagnetic field might be detected in Hymenoptera: a ferromagnetic hypothesis based on magnetic particles (also called particle-mediated mechanism) (Shaw et al., 2015) and a biochemical hypothesis based on light-sensitive cryptochromes (also called radical-pair mechanism) (Hore and Mouritsen, 2016). For both theoretical mechanisms, there is empirical evidence in the animal kingdom. The biochemical hypothesis suggests that the geomagnetic field is detected by a light-dependent biochemical reaction. The most promising candidate molecule that provides a suitable radical pair is cryptochrome. It has been shown to be crucial for magnetoreception in birds (Hore and Mouritsen, 2016) and fruit flies (Gegear et al., 2008). Additionally, the magnetoreception in many animals is light and light-spectrum dependent (Guerra et al., 2014; Hore and Mouritsen, 2016; Vácha, 2006).

However, Hymenoptera, like *Cataglyphis*, do not possess a light sensitive-cryptochrome that would match the requirements for the radical-pair mechanism (Yuan et al., 2007). Additionally, some Hymenoptera can use their magnetic sense also in complete darkness (Camlitepe and Stradling, 1995; Schmitt and Esch, 1993). This makes it unlikely that *Cataglyphis* uses a magnetic compass mediated by a radical-pair mechanism. However, we cannot exclude that there might be a completely different protein serving a similar function in Hymenoptera.

Magnetic orientation with the help of particles has already been described in small, single-cell organisms (Blakemore, 1975). Similar to this, the ferromagnetic hypothesis suggests that the geomagnetic field is sensed through sensory neurons that possess ferromagnetic particles. While moving through the geomagnetic field, the mechanically coupled particles would create a neuronal stimulus, e.g., activating mechanosensitive ion channels (Clites and Pierce, 2017; Shaw et al., 2015). Such ferromagnetic particles have been found in many Hymenoptera (Abraçado et al., 2008; Eder, 2012; Riveros et al., 2014; Wajnberg et al., 2010, 2017). However, whether they are functionally linked to ion channels or other neuronal structures is not known.

Hymenoptera are able to get unambiguous directional information from their magnetic compass (Lambinet et al., 2017). The discrimination between magnetic north and south can be achieved in two ways (Figure 5): the magnetic compass is either polarity sensitive or an inclination compass (Fleischmann et al., 2020b). A particle-mediated mechanism enables an animal to detect the polarity of the geomagnetic field, while the radical-pair mechanism would be able to detect the inclination – the angle of the geomagnetic vector relative to earth's gravitational vector (Clites and Pierce, 2017).

Since honey bees are able to detect the polarity (Lambinet et al., 2017), it seems likely that *Cataglyphis* might do so, too. To test whether *Cataglyphis* has a polarity-sensitive compass, the vertical and the horizontal components of the GMF have to be manipulated separately using 3D Helmholtz coils (Figure 5).

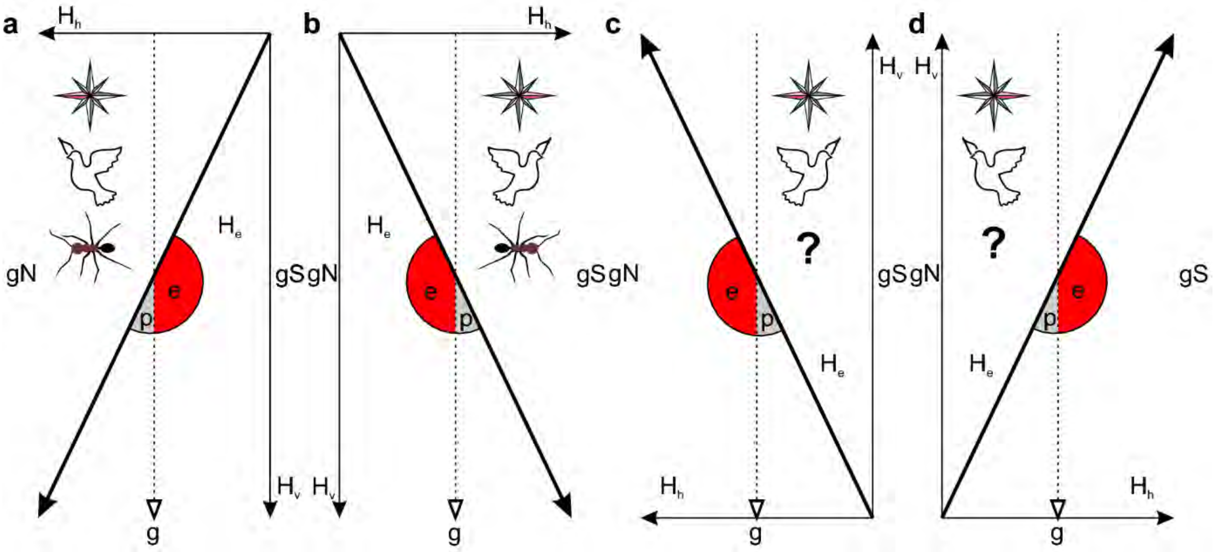

Figure 5 Polarity sensitive *versus* inclination compass. A polarity-sensitive compass and an inclination compass will lead to different outcomes under different experimental conditions. **a)** The natural magnetic field is composed of the horizontal component (H_h) and the vertical component (H_v), providing the total magnetic field vector (H_e) (shown for the magnetic field on the northern hemisphere). **b)** When H_h is reversed, both polarity and inclination of H_e are changed. **c)** When only H_v is reversed, the inclination of H_e is changed while its polarity remains the same. If animals follow this change, they use an inclination compass. An inclination compass measures the angle between the H_e and gravity (g), and distinguishes between poleward (p) and equatorward (e). Several bird species possess an inclination compass. **d)** When both H_h and H_v are reversed, the polarity of H_e is changed while its inclination remains the same. Animals following this change use an polarity sensitive compass. For desert ants, it is not known whether they possess a polarity-sensitive or an inclination compass. (Figure from Fleischmann et al., 2020).

Unfortunately, *Cataglyphis* do not perform learning walks in complete darkness, testing for light-dependency of the magnetic compass with a reliable behavioral readout is difficult. However, there are several experiments that test for different mechanism: The light conditions of the ants could be altered during leaning walks to exclude wavelengths that are necessary for a light-dependent magneto sensing mechanism. In birds, it was shown that wavelengths below 590 nm are necessary for magnetic orientation (Wiltschko and Wiltschko, 2001, 2002, 2005).

Additionally, it has been shown that broadband radiofrequencies can disrupt orientation based on a radical-pair magnetoreception mechanism (Granger et al., 2022; Leberecht et al., 2022; Nießner and Winklhofer, 2017; Ritz et al., 2004). If

Cataglyphis would be presented with broadband radio frequencies during learning and should they use a radical-pair magnetoreception mechanism, their magnetic orientation during pirouettes would be disrupted. This has also been proposed for honeybees (Válková and Vácha, 2012).

To test for the dependency of magnetic orientation based on magnetic particles a magnetic pulse experiment, as has been performed in other arthropods (Ernst and Lohmann, 2016) and birds (Wiltschko et al., 1994; Wiltschko and Wiltschko, 2005), could be performed. Using a strong magnetic pulse on the ants might either re-magnetize their magnetic particles or dislocate the magnetic particles of the sensory cells, while a radical-pair mechanism would not be influenced by this treatment. If the magnetic compass of *Cataglyphis* is indeed mediated by ferromagnetic particles, it begs the question of where these particles come from. The ants could either biomineralize them on their own or obtain them from the environment. It has been shown that leaf-cutter ants need to have had contact with their natural soil in order to use their magnetic compass. This indicates that they might take up magnetic particles from the soil (Riveros et al., 2014).

7.4 The Antennae as a Potential Magnetic Sensor

Despite much research being done on the magnetic sense, the sensory organ and cellular transduction mechanism for magnetoreception remains elusive in any animal (Nordmann et al., 2017). Additionally to being involved in flight control, and being sensitive to sound, wind, and gravitation, Johnston’s organ of ants might have an additional function as a magnetic compass (Chapter 4: Grob et al., 2021c). The ants’ antenna and especially the Johnston’s organ provide a plausible candidate for the magnetic compass in insects. It has already been shown, that the antennae play a crucial role in the magnetic compass of the monarch butterfly (Guerra et al., 2014). To detect magnetic fields in the Johnston’s organ, one possible mechanism is that the antenna gets deflected by magnetic forces. In a similar way, honeybees detect electromagnetic fields. In bees, Johnston’s organ detects slight movements of the flagellum by electrostatic forces (Greggers et al., 2013). Alternatively, magnetic particles inside the Johnston organ could mechanically open iron channels of receptor neurons. The ring-like arrangement of the scolopidia in *Cataglyphis* (Chapter 4: Grob et al., 2021c) might promote polarity sensitive magnetoreception. Due to the different location of the scolopidia, some of the sensory neurons would always be maximally activated depending on the their position in the magnetic field. Interestingly, each scolopidium of the *Cataglyphis* Johnston organ houses three receptor neurons (Chapter 4: Grob et al., 2021c). In *Drosophila* one of these three receptor neurons is sound-sensitive and another one is wind-sensitive (Ishikawa et al., 2020). This might indicate that the third receptor neuron in each scolopidium is sensitive to a third modality, potentially the magnetic field.

Additionally, in the antennae of stingless bees and ants a sufficient amount of magnetic material has been found to potentially enable magnetoreception (de Oliveira et al., 2010; Lucano et al., 2006). Another indication for the involvement of the Johnston's organ in magnetoreception is, that the magnetic sense of Hymenoptera seems to be entangled with the gravitational sense (Lindauer and Martin, 1968; Martin and Lindauer, 1977). Since the Johnston's organ is involved in both graviception (Vowles, 1954b) and might be involved in sensing the magnetic field (de Oliveira et al., 2010), an entanglement of these two cues could be expected. Similar interactions have been described between hearing and the reception of electromagnetic fields which are also detected by the same organ (Greggers et al., 2013).

Future experiments might shed light on the presence of magnetic particles in the ants' antennae and their relation to the Johnston's organ. Similar to the experiments performed with electric fields in bees (Greggers et al., 2013), physiological methods might even allow to record from Johnston's organ afferents in a changing magnetic field.

7.5 Closing Remarks and Outlook

The present doctoral thesis shows how *Cataglyphis* ants are capable of calibrating their compass systems during the transition phase from interior worker to outdoor forager. The key findings of my doctoral thesis and their importance for the understanding of animal navigation are summarized in the following paragraph.

Cataglyphis desert ants are marvelous navigators, with a brain that, while tiny, holds impressive navigational computation power and is well adapted to successfully accomplish the navigational needs of the ants (Chapter 4: Grob et al., 2021b). Desert ants' primary compass during their far-reaching foraging trips is their celestial compass

(Wehner, 2020). However, due to the ever-changing position of orientation clues in the sky, the ants need to calibrate their compass systems before foraging. To do so, the ants perform well structured learning walks (Fleischmann et al., 2017; Zeil and Fleischmann, 2019). Manipulating the celestial orientation cues during initial learning walks has no influence on the directedness of the look-back behavior. This shows that the celestial compass cues do not guide the ants during initial learning walks (Chapter 2: Grob et al., 2017). Surprisingly, *C. nodus* uses the earth's magnetic field as a compass during this early learning phase (Chapter 3: Fleischmann et al., 2018a). While not guiding the ants during their first walks outside of the nest, excluding the ants from perceiving the natural polarization pattern of the skylight has significant consequences on learning-related plasticity in the ants' brain (Chapter 2: Grob et al., 2017). Only if the ants are able to perform their learning-walk behavior under a skylight polarization pattern that changes throughout the day, plastic changes in the mushroom bodies and the central complex of the *Cataglyphis* brain are induced (Chapter 2: Grob et al., 2017 and Chapter 6: Grob et al., 2022). This underlines the importance of learning walks for calibrating the celestial compass. The magnetic compass might provide the necessary stable earthbound reference system for the ants to calibrate their celestial compass and learn the position of landmark information. Neuronal afferents of the mechanosensitive Johnston's organ in the ant's antennae converge in tight apposition with visual information from the ocelli (Chapter 4: Grob et al., 2021c). This makes the ants' antennae an interesting candidate for studying the sensory bases of compass calibration in *Cataglyphis* ants.

During my doctoral thesis, I was able to employ a wide range of state-of-the-art techniques from different disciplines in biology to gain a deeper understanding of how navigational information is acquired, memorized, used, and calibrated. These results underline the importance and advantages of *Cataglyphis* as an experimental model for orientation and navigation, even when compared to better known far distance navigators like birds or butterflies. Our neuroethological approach opened up a broad field of research questions, from which I will discuss three major experiments.

1) While the plastic neuronal changes during learning walk strongly suggest the calibration of the solar ephemeris during initial learning walks, we are still missing the behavioral proof. To test this, future experiments should train the ants under different conditions during initial learning walks - the natural changing sky, artificially stable skylight cues, and as a reference useless

magnetic field – and test their ability to time compensate their celestial compass during a displacement experiment. To do this, the experimental design established by Fleischmann et al. (2018b) might be of great use.

2) The discovery of the earth’s magnetic field as a compass in *Cataglyphis* opens up many questions. Several future experiments (described in more detail above) can shed light on the nature of the magnetic compass in *C. nodus*: Is the magnetic compass particle-based, or does it use a light-dependent mechanism? Is it sensitive to the polarity of the magnetic field or an inclination compass? Additionally, it is still unknown where the magnetic compass in ants is located. It has been suggested that the antennae of insects might play a crucial role in magnetoreception. To test this, the antennae of naïve ants could be manipulated during learning walks, either with paint or using ferromagnetic particles. In combination with electrophysiological studies of the antennal output in changing magnetic fields, will provide new insides into the role of the insect antennae in magnetoreception. Follow-up questions, like how the magneto sensitive receptor neurons are connected to the central nervous system and how the magnetic compass information is integrated into the central compass for path integration, will have to be investigated using a combination of anatomical and physiological studies.
3) Why the ants switch from their apparently very precise and highly accurate innate magnetic compass to their celestial compass remains a major open question. The celestial compass needs extensive calibration and, if indeed is calibrated using the magnetic field, cannot be more accurate than the magnetic compass. Might the mechanism for magnetoreception be too slow for their high-speed foraging runs? Might it be too energy-consuming? Might it seize too much computational brainpower? Or might the organ that is used for sensing magnetic field serve a different function during foraging? Unless we get a better understanding of the neuronal mechanism underlying magnetoreception, this question will be hard to answer. However, studying the ants’ behavior throughout their foraging careers – from initial learning walks, to successful foragers, from learning a new profitable food side, to re-calibrating their navigational systems – will provide a better understanding of the flexibility of cue hierarchy in their navigational system.

Their well-structured and well-studied behaviors that are performed in an easily observable range combined with the straightforward ontogeny and our knowledge of the neuronal mechanism underlying the navigational capabilities make *Cataglyphis* ants the perfect animal model for studying the acquisition and calibration of navigational strategies. Being able to study these small expert navigators in their natural habitat was and is truly awe-inspiring to me.

“Finally, there remains for mention a study of the senses of ants with particular emphasis on the question of how they find their way back to the nest. Among many other facts it is shown that there are great differences in visual power in ants, which include visual, oligovisual, and olfactory (blind) types. In reality the ant, like any higher animal, combines for its guidance all the sensory data at its disposal, without reckoning the internal impulses which arise spontaneously in association with earlier impressions preserved by memory.”

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Affidavit

I hereby confirm that my thesis entitled “The Function of Learning Walks of Cataglyphis Ants: Behavioral and Neuronal Analyses” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Die Funktion der Lernläufe in *Cataglyphis* Ameisen: eine Studie des Verhaltens und der neuronalen Auswirkungen” eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

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"Alone we can do so little; together we can do so much."

Helen Keller

Curriculum vitae with list of publications

Publications

Peer-reviewed Articles

2022

Grob, R., Holland Cunz, O., Grübel, K., Pfeifer, K., Rössler, W., Fleischmann, P. N. (2022). **Rotation of Skylight Polarization During Learning Walks is Necessary to Trigger Neuronal Plasticity in *Cataglyphis* Ants.** *Proc. Royal Soc. B*. 289: 20212499. doi: 10.1098/rspb.2021.2499

2021

Grob, R., Heinig, N., Grübel, K.,.Rössler, W., Fleischmann, P. N (2021). **Sex-specific and caste-specific brain adaptations related to spatial orientation in *Cataglyphis* ants.** *J. Comp. Neurol.* 529:18, 3882–3892. doi: 10.1002/cne.25221.

Fleischmann, P. N., **Grob, R.**, Rössler, W. (2021). **Magnetosensation during re-learning walks in desert ants (*Cataglyphis nodus*).** doi: 10.1007/s00359-021-01511-4.

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2020

Fleischmann, P. N., **Grob, R.**, and Rössler, W. (2020). **Magnetoreception in Hymenoptera: importance for navigation.** Animal Cognition. 23:6, 1051-1061. doi: 10.1007/s10071-020-01431-x.

2019

Grob, R., Fleischmann, P. N., and Rössler, W. (2019). **Learning to navigate – how desert ants calibrate their compass systems.** Neuroforum. doi: 10.1515/nf-2018-0011.

2018

Fleischmann, P.N., **Grob, R.**, Müller, V. L., Wehner, R., and Rössler, W. (2018). **The Geomagnetic Field Is a Compass Cue in *Cataglyphis* Ant Navigation.** *Curr. Biol.* 28 :9, 1440–1444 doi: 10.1016/j.cub.2018.03.043.

2017

Grob, R., Fleischmann, P. N., Grübel, K., Wehner, R., and Rössler, W. (2017). **The Role of Celestial Compass Information in *Cataglyphis* Ants during Learning Walks and for Neuroplasticity in the Central Complex and Mushroom Bodies.** *Front. Behav. Neurosci*. 11:226. doi: 10.3389/fnbeh.2017.00226.

Fleischmann, P. N., **Grob, R.**, Wehner, R., and Rössler, W. (2017). **Species-specific differences in the fine structure of learning walk elements in *Cataglyphis* ants.** *J. Exp. Biol.* 220, 2426–2435. doi:10.1242/jeb.158147.

Popular Scientific Articles

2020

Fleischmann, P. N., **Grob, R.**, and Rössler, W. (2020). **Kompass im Kopf - Wie Wüstenameisen lernen heimzukehren (Ant compass – how desert ants learn to navigate).** *Biol. Unsere Zeit* 2/2020:50, 100–109. doi: 10.1002/ biuz.202010699.

Talks and Poster Presentations

2021

Grob, R., Holland Cunz, O., Grübel, K., Pfeiffer, K., Rössler, W., Fleischmann, P. N. (2021)
Acquisition of the Solar Ephemeris: Rotation of the Sky Triggers Neuronal Plasticity in *Cataglyphis* Ants. 113th Annual Meeting of the German Zoological Society – DZG21, Würzburg (Germany), 30th of August – 3rd of September. (Talk)

Grob, R., Tritscher, C., Grübel, K., Stigloher, C., Groh, C., Fleischmann, P. N., Rössler, W. (2021)
The Johnston's organ of desert ants and its central projections. 14th Göttingen Meeting of the German Neuroscience Society, Göttingen (Germany), 22nd – 30th of March. (Poster)

Grob, R., Tritscher, C., Grübel, K., Stigloher, C., Groh, C., Fleischmann, P. N., Rössler, W. (2021)
The Johnston's organ of desert ants and its central projections. 14th Göttingen Meeting of the German Neuroscience Society, Göttingen (Germany), 22nd – 30th of March. (Talk)

2019

Grob, R., Fleischmann, P. N., Wehner, R., and Rössler, W. (2019).
Setting Your Compass – how desert ants learn to navigate. 14th Eureka! International Symposium, University of Würzburg, Würzburg, 9th – 10th of October. (Poster)

Grob, R., Fleischmann, P. N., Wehner, R., and Rössler, W. (2019).
Learning to navigate – how desert ants calibrate their compass systems. 30th NeuroDoWo19, University of Würzburg, Würzburg, 28th – 31th of August. (Talk)

Grob, R. (2019) **Calibrating the celestial compass.** 2nd Retreat of the Institute of Behavioral Physiology & Sociobiology, University of Würzburg, Würzburg, 10th of Mai. (Talk)

Grob, R., Fleischmann, P. N., Müller, V. L., Wehner, R., and Rössler, W. (2019).
The earth's magnetic field is an early compass in desert ants. 10th Royal Institute of Navigation Conference on Animal navigation (RIN19), Royal Holloway College, London (UK), 10th – 12th of March. (Poster)

Grob, R., Fleischmann, P. N., Grübel, K., Wehner, R., and Rössler, W. (2019).
Compass Systems During Ant Learning Walks: The Role of Celestial Cues for Initial Compass Calibration in *Cataglyphis* Ants. 13th Göttingen Meeting of the German Neuroscience Society, Göttingen (Germany), 20th – 23th of March. (Talk)

2018

Grob, R., Fleischmann, P. N., Müller, V. L., Wehner, R., and Rössler, W. (2018).
Navigating with the sixth sense - how desert ants set their compass systems. 13th Eureka! International Symposium, Würzburg (Germany), 10th – 11th of October. (invited Talk)

Grob, R., Fleischmann, P. N., Müller, V. L., Wehner, R., and Rössler, W. (2018).
The role of a geomagnetic compass during learning walks of *Cataglyphi*s desert ants. "Sensory Ecology" 2018, Lund (Sweden), 23th of September– 07th of October. (Poster)

Grob, R., Fleischmann, P. N., Müller, V. L., Wehner, R., and Rössler, W. (2018).
The role of a geomagnetic compass for early learning behavior in desert ants. Annual meeting of the German Zoological Society (DZG), Greifswald, Germany, 11th – 14th of September. (Talk)

Grob, R., Fleischmann, P. N., Grübel, K., Wehner, R., and Rössler, W. (2018).
Setting Your Compass - Behavioral and Neuronal Effects of Skylight Manipulation during the Learning Walks of *Cataglyphis* Ants. ANN Spring Meeting 2018, Altleiningen (Germany), 27th – 29th of March. (Talk)

2017

Grob, R., Fleischmann, P. N., Grübel, K., Wehner, R., and Rössler, W. (2017). **Skylight-dependent behavioral changes and neuronal plasticity in visual pathways of *Cataglyphis* desert ants.** FENS-Hertie Winter School: Neural control of behaviour - Series 1: Navigation, Obergurgl (Austria), 10th – 16th of December. (Poster)

Grob, R., Fleischmann, P. N., Grübel, K., Wehner, R., and Rössler, W. (2017). **Learning walks are crucial – Behavioral and Neuronal Effects of Skylight Manipulation.** New Frontiers in Insect Navigation, Würzburg (Germany), 16th – 17th of November. (Talk)

SCIENTIFIC MEETINGS

2019 Co-Organization of the 30th Neurobiology Doctoral Students Workshop "**NeuroDoWo19**", Residence Würzburg, JMU Würzburg, Germany

2017 Co-Organization of the Symposium "**New Frontiers in Insect Navigation**", Biocentre, JMU Würzburg, Germany (with Dr. Pauline Fleischmann)

Würzburg, 15/02/2022

Appendix

“Dissertation Based on Several Published Manuscripts“

Statement of individual author contributions and of legal second publication rights

(If required please use more than one sheet)

Publication (complete reference): Grob, R*, Fleischmann, P. N*, Grübel, K., Wehner, R., Rössler, W. The Role of Celestial Compass Information in *Cataglyphis* Ants during Learning Walks and for Neuroplasticity in the Central Complex and Mushroom Bodies. 2017 *Frontiers in Behavioral Neuroscience*, 11, 226.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	PNF RG	WR PNF	RG WR	RW KG	
Data Collection	RG	PNF	KG		
Data Analysis and Interpretation	RG*	PNF*	WR	RW	KG
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 RG* RG* RG* RG	 PNF* PNF* PNF* PNF	 WR WR WR	 RW RW RW	

Explanations (if applicable):

*These authors have contributed equally to this work.

Publication (complete reference): Fleischmann, P. N*, Grob, R*, Müller, V. M., Wehner, R.$^{+}$, Rössler, W.$^{+}$ The Geomagnetic Field is a Compass Cue in *Cataglyphis* Ant Navigation. 2018, Current Biology 28, 1440–1444

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	PNF* PNF*	RG* RG*	WR^{+} VLM	RW^{+} WR	VLM
Data Collection	PNF*	RG*			
Data Analysis and Interpretation	PNF*	RG*	WR^{+}	RW^{+}	VLM
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 PNF* PNF* PNF* PNF*	 RG* RG* RG* RG*	 WR^{+} VLM WR^{+}	 RW^{+} WR^{+} RW^{+}	 VLM RW^{+} VLM

Explanations (if applicable):

*These authors have contributed equally to this work.
$^{+}$These authors share a senior authorship.

Publication (complete reference): Grob R, Tritscher C, Grübel K, Stigloher C, Groh C, Fleischmann PN, Rössler W (2021), Johnston's organ and its central projections in Cataglyphis desert ants. J Comp Neurol. 2020;1–18.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	RG	WR	PNF	CG	CS
Methods Development	RG	CG	CS	CT	KG
Data Collection	RG	CT	KG		
Data Analysis and Interpretation	RG	CT	WR	PNF	CG
Manuscript Writing					
Writing of Introduction	RG	WR	PNF		
Writing of Materials & Methods	RG	WR	PNF		
Writing of Discussion	RG	WR	PNF		
Writing of First Draft	RG				

Explanations (if applicable):

Publication (complete reference): Grob, R., Heinig, N., Grübel, K., Rössler, W., & Fleischmann, P. N. (2021). Sex-specific and caste-specific brain adaptations related to spatial orientation in *Cataglyphis* ants. Journal of Comparative Neurology, 529(18), 3882-2892.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	RG	PNF[+]	WR[+]		
Methods Development	RG	KG	PNF[+]	WR[+]	
Data Collection	RG	NH			
Data Analysis and Interpretation	RG	NH	PNF[+]	WR[+]	KG
Manuscript Writing					
Writing of Introduction	RG	PNF[+]	WR[+]		
Writing of Materials & Methods	RG	PNF[+]	WR[+]		
Writing of Discussion	RG	PNF[+]	WR[+]		
Writing of First Draft	RG				

Explanations (if applicable):

[+] These authors share a senior authorship.

Publication (complete reference): Grob R, Holland Cunz O, Grübel K, Pfeiffer K, Rössler W^{+}, Fleischmann PN^{+}, Rotation of Skylight Polarization During Learning Walks is Necessary to Trigger Neuronal Plasticity in *Cataglyphis* Ants. Proceedings of the Royal Society B. 289: 20212499.

Participated in	**Author Initials,** Responsibility decreasing from left to right					
Study Design	RG	PNF^{+}	WR^{+}			
Methods Development	RG	CG	PNF^{+}	WR^{+}	KP	
Data Collection	RG	OHC	PNF			
Data Analysis and Interpretation	RG	OHC	CG	PNF^{+}	WR^{+}	KP
Manuscript Writing						
Writing of Introduction	RG	PNF^{+}	WR^{+}	KP		
Writing of Materials & Methods	RG	PNF^{+}	WR^{+}	KP		
Writing of Discussion	RG	PNF^{+}	WR^{+}	KP		
Writing of First Draft	RG					

Explanations (if applicable):

$^{+}$These authors share a senior authorship.

The doctoral researcher confirms that she/he has obtained permission from both the publishers and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Robin Grob Würzburg

Doctoral Researcher's Name Date Place Signature

Prof. Dr. Wolfgang Rössler 09.02.2022 Würzburg

Primary Supervisor's Name Date Place Signature

“Dissertation Based on Several Published Manuscripts“

Statement of individual author contributions to figures/tables/chapters included in the manuscripts

(If required please use more than one sheet)

Publication (complete reference): Grob, R*, Fleischmann, P. N*, Grübel, K., Wehner, R., Rössler, W. The Role of Celestial Compass Information in *Cataglyphis* Ants during Learning Walks and for Neuroplasticity in the Central Complex and Mushroom Bodies. 2017 *Frontiers in Behavioral Neuroscience*, 11, 226.

Figure	**Author Initials,** Responsibility decreasing from left to right				
1	RG*	PNF*			
2	RG*	PNF*			
3a,b	WR	KG			
3	RG*	PNF*	WR		
4	RG*	PNF*			
5	RG*	PNF*			
6	RG*	PNF*			
ST1	RG*	PNF*			

Explanations (if applicable):

*These authors have contributed equally to this work.

Publication (complete reference): Fleischmann, P. N*, Grob, R*, Müller, V. M., Wehner, R.$^{+}$, Rössler, W.$^{+}$ The Geomagnetic Field is a Compass Cue in *Cataglyphis* Ant Navigation. 2018, Current Biology 28, 1440–1444

Figure	**Author Initials,** Responsibility decreasing from left to right				
Graphical Abstract	RG*	PNF*			
1	PNF*	RG*			
2	PNF*	RG*			
3	PNF*	RG*			
4	PNF*	RG*			
Video S1	RG	PNF			

Explanations (if applicable):

*These authors have contributed equally to this work.

Publication (complete reference): Grob R, Tritscher C, Grübel K, Stigloher C, Groh C, Fleischmann PN, Rössler W (2021), Johnston's organ and its central projections in Cataglyphis desert ants. J Comp Neurol. 2020;1–18.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
Graphical Abstract	RG				
1	RG	CT	CS		
2	RG	CT			
3	RG	CT			
4	RG	CT			
5	RG	CT			
6	RG	KG	WR		
7	KG	RG	WR		
8	KG	RG	WR		
9	KG	RG	WR		
10	RG	CT	CS		
T1	RG	KG			

Explanations (if applicable):

Publication (complete reference): Grob, R., Heinig, N., Grübel, K., Rössler, W., & Fleischmann, P. N. (2021). Sex-specific and caste-specific brain adaptations related to spatial orientation in Cataglyphis ants. Journal of Comparative Neurology, 529(18), 3882-2892.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	RG	PNF	KG	NH	
2	RG	PNF			
3	RG	PNF			
4	RG	PNF	KG	NH	
5	RG	PNF	KG		
T1	RG				
T2	RG	NH			
T3	RG	KG			

Explanations (if applicable):

Publication (complete reference): Grob R, Holland Cunz O, Grübel K, Pfeiffer K, Rössler W$^+$, Fleischmann PN$^+$, Rotation of Skylight Polarization During Learning Walks is Necessary to Trigger Neuronal Plasticity in *Cataglyphis* Ants. Proceedings of the Royal Society B. 289: 20212499.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	RG	OHC	KG		
2	RG				
3	RG				
ST1	RG	PNF			

I also confirm my primary supervisor’s acceptance.

Robin Grob		Würzburg	
Doctoral Researcher’s Name	Date	Place	Signature