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Straight-line orientation in South African ball-rolling dung beetles

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Keep it rolling

Straight-line orientation in South African ball-rolling dung beetles

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Keep it rolling

Straight-line orientation in South African ball-rolling dung beetles

Lana Khaldy



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DOCTORAL DISSERTATION

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Abstract Representing a substantial range and variety in morphological and ecological niche, found on all continents of the globe (except for the Antarctic), <i>the ball-rolling dung beetles</i> provide an excellent model in which to study <i>the heading direction network</i> and the factors by which it is influenced. As soon as a ball-rolling dung beetle has located a fresh dung pile to feed on, it immediately starts shaping a piece of dung into a ball, rolling it away from the dung pat in as straight of a trajectory as the terrain allows. This <i>straight-line orientation behaviour</i> is thought to be a strategy to escape the fierce competition of dung at the pile. By investigating how <i>size</i> (Paper I), <i>ecological niche</i> , <i>phylogeny</i> (Paper II and Paper III) and <i>visual conditions</i> (Paper IV) influence this relatively straightforward orientation behaviour, I explore the orientation challenges faced, and the solutions presented. In the first paper (Paper I), I investigated the effect of directional error on straight-line orientation in two differently sized beetles, and concluded that the directional error that unavoidably accumulates over a given distance as the beetle travels, is inversely proportional to the step size of the animal. Next (Paper II), I investigated straight-line orientation in a diurnal woodland-living ball-rolling species. In this study I demonstrated that the woodland-living species, present in habitats of densely packed trees and tall grass, relies predominantly on directional information from the celestial pattern of polarised light. This stands in contrast to all previous observations on diurnal ball-rolling beetles, where the sun has been demonstrated as the predominant source of directional information in their heading direction networks. In the third paper (Paper III) I continued to explore the relative weighting of directional information in three species of ball-rolling South African dung beetles from three different tribes living within the same savanna biome, but in different habitat types. In this study I found that species <i>within</i> a tribe share the same orientation strategy, but that this strategy differs <i>across</i> tribes. In my final paper (Paper IV), I further explored the weight relationship of directional information from the sun (simulated by a green LED) and the celestial polarisation pattern (simulated by an overhead band of polarisation) in the heading direction network of the beetle. I concluded that the directional information conveying the highest certainty at a given moment in time is afforded the greatest weight in the heading direction network of the animal. With my work, I hope to provide an insight to the dynamic nature of the biological compass and its ability to change and adapt to different visual environments.		
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Keep it rolling

Straight-line orientation in South African ball-rolling
dung beetles

Lana Khaldy



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Scientific Papers

- I. **Khaldy L, Peleg O, Tocco C, Mahadevan L, Byrne M, Dacke M. (2019).** The effect of step size on straight-line orientation. *J R Soc Interface* **16**, 20190181. <https://doi.org/10.1098/rsif.2019.0181>
- II. **Khaldy L, Tocco C, Byrne M, Baird E, Dacke M. (2019).** Straight-line orientation in the woodland-living beetle *Sisyphus fasciculatus*. *J Comp Physiol A* **206**, 327-335. <https://doi.org/10.1007/s00359-019-01331-7>
- III. **Khaldy L, Tocco C, Byrne M, Dacke M. (2021).** Compass cue integration and its relation to the visual ecology of three tribes of ball-rolling dung beetles. *Insects* **12**, 526. <https://doi.org/10.3390/insects12060526>
- IV. **Khaldy L, Foster JJ, Yilmaz A, Belušič G, Gagnon Y, Tocco C, Byrne M, Dacke M.** The interplay of directional information provided by unpolarised and polarised light in the heading direction network of *Kheper lamarcki*. (*Manuscript submitted*).

Author Contributions

- I. L.K., O.P., C.T., M.B. and M.D. conducted experiments; L.K., O.P., L.M. and M.D. designed experiments; L.K., O.P. and M.D. analysed the data; L.K. drafted the manuscript; all authors revised the manuscript.
- II. L.K. and C.T. conducted experiments; L.K. and M.D. designed experiments; L.K. analysed the data; L.K. drafted the manuscript; all authors revised the manuscript.
- III. L.K and C.T. conducted experiments; L.K. designed experiments; L.K. analysed the data; L.K. drafted the manuscript; all authors revised the manuscript.
- IV. L.K conducted behavioural experiments; A.Y. and G.B. collected physiology data; C.T. and M.B. collected and transported animals; L.K., J.F., Y.G. and M.D. designed behavioural experiments; L.K. analysed the behavioural data; A.Y. and G.B. analysed the physiology data; L.K. drafted the manuscript; all authors have revised the current draft of the manuscript.

Scientific Papers not included in this Thesis

- el Jundi B, Warrant EJ, Byrne MJ, **Khaldy L**, Baird E, Smolka J, Dacke M. (2015). Neural coding underlying the cue preference for celestial orientation. *Proc Natl Acad Sci USA* **112**, 11395-11400. <https://doi.org/10.1073/pnas.1501272112>.
- el Jundi B, Foster J, **Khaldy L**, Byrne MJ, Dacke M, Baird E. (2016). A snapshot-based mechanism for celestial orientation. *Curr Biol* **26**, 1456–62. <https://doi.org/10.1016/j.cub.2016.03.030>.
- Foster JJ, el Jundi B, Smolka J, **Khaldy L**, Nilsson D-E, Byrne MJ, Dacke M. (2017). Stellar performance: Mechanisms underlying Milky Way orientation in dung beetles. *Phil Trans R Soc Lond B Biol Sci* **372**, 20160079. <https://doi.org/10.1098/rstb.2016.0079>.
- Foster JJ, Kirwan JD, el Jundi B, Smolka J, **Khaldy L**, Baird E, Byrne MJ, Nilsson D-E. (2019). Orienting to polarized light at night – Matching lunar skylight to performance in a nocturnal beetle. *J Exp Biol* **222**, 1–10. <https://doi.org/10.1242/jeb.188532>.
- Foster JJ, Tocco C, Smolka J, **Khaldy L**, Baird E, Byrne M, Nilsson D-E, Dacke M (2021). Light pollution forces a change in dung beetle orientation behaviour. *Curr Biol* **31**, 1-8. <https://doi.org/10.1016/j.cub.2021.06.038>

Popular summary

Although unpleasant to our senses, fresh dung is the best part of the day to many insects. Among these are the *South African ball-rolling dung beetles*. When it is time to feed, these insects emerge from the ground and fly to the nearest suitable dung pat. Once it has (crash)landed on or nearby the dung pile (the landing of a dung beetle is not a particularly graceful one), it quickly shapes a portion of the dung into a ball. There can be hundreds of beetles on the dung pat, all trying to get their share of the food. Some of these do not bother forming balls of their own but will rather try to hijack those of others. Thus, once the ball has been shaped, it is crucial to quickly get away from the chaos at the pile. One way to do this is to roll your ball away along a path as straight as the terrain allows, maximising the distance gained to your competitors with every step taken. To steer straight across the savanna, the beetles integrate directional information from different celestial cues, such as the position of the sun or the orientation of the skylight polarisation pattern (a light pattern in the sky created by the scattering of sunlight) into their *internal compass*.

As a dung beetle researcher, it is not uncommon to find yourself in the scorching heat of the savanna, staring at a pile of dung. After many hours of doing this myself, I started to pay attention to the wide variation in size of ball-rolling dung beetles, feeding from the same dung pat. When a beetle is moving its limbs, mechanical and sensory noise is generated, producing overall fluctuations in the forward motion of the beetle, causing it to deviate from its straight path. As beetles of different size have different step lengths, this made me wonder how the size of a beetle affects its ability to steer straight. In **Paper I**, I answered this by comparing the *straight-line orientation strategy* of two species of ball-rolling dung beetles that differ greatly in size. I found that the noise generated over a given distance is inversely proportional to the step size of the animal. This means that over the same distance, smaller sized beetles –that take many more steps than the larger ones– end up having a more tortuous roll path. Interestingly, in their natural setting on the savanna, both beetles take an equal number of steps before burying down, but because of the noise generated, smaller beetles end up radially closer to the dung pile compared to larger beetles.

Many of the ball-rolling beetles on the savanna will primarily steer by the sun, but what about beetles living in more cluttered environments? In **Paper II** I explored the straight-line orientation behaviours of a dung beetle species living in regions where the sun is frequently hidden behind clouds or the overhead canopy. I found that beetles inhabiting this environment primarily rely on the polarised skylight pattern to guide their paths. This could suggest that the visual environment of dung beetles plays a role in the design of the neuronal compass. However, my results were only demonstrated in one species. Therefore, following my findings from Paper II, I set out to explore the

role of directional information from the sun and the skylight polarisation pattern in the *heading direction network* of beetles across different tribes, living within the same region. In **Paper III** I found that each of the three tribes tested presented a different strategy; the first tribe relied predominantly on the sun for directional guidance, the second tribe relied on the pattern of polarised skylight, while the third tribe did not appear to favour either of these two cues. This suggests that in these three tribes of beetles, the different weights given to these two sources of directional information is dictated by their phylogeny, rather than their visual ecology.

It is important to note, that a beetle relying predominantly on the sun, does not suddenly start to roll in circles as soon as passing clouds or branches of a tree shades the sun. Instead, these beetles rather rely on the 'second most popular' cue for directional guidance: the pattern of polarised skylight. But what is it that dictates when this change in directional guidance should be made? In **Paper IV** I built a setup presenting a simulated sun together with a simulated skylight polarisation pattern. By changing the properties of these two cues (for instance by changing their relative intensity) and analysing the beetle's response, I found that the more unreliable a cue appears to the beetle, the less weight is given to it in its heading direction network.

Through my four papers I hope to have demonstrated the dynamic nature of the heading direction network of the ball-rolling dung beetles that allow these incredible animals to steer straight across most continents and vegetation types of the world.

Populär Sammanfattning

En ordentlig hög dynga är den absoluta höjdpunkten på dagen för många insekter, inte minst för de *sydafrikanska boll-rullande dyngbaggar*. När det är matdags, gräver sig dessa stora insekter upp ur marken, faller ut sina vingar och flyger till en lämplig dynghög. Efter att ha (krasch)landat på eller vid denna tillfälliga uteservering (en dyngbaggens landning är inte den mest graciösa), börjar skalbaggen snart skulptera sig en boll. Ofta finns det upp till hundra dyngbaggar på en dynghög, alla med det gemensamma målet att äta sig mätta. Några av dessa formar inte nödvändigtvis sina egna bollar, utan provar att stjäla andras. Så snart en dyngbagg färdigställt sin boll gäller det därför att få iväg den från kaoset runt dynghögen så snabbt som möjligt. Genom att hålla en stabil kurs med sin runda matlåda maximerar bollägaren det avstånd den kan lägga mellan sig och konkurrenterna med vart fotsteg den tar. För att styra rakt använder sig dyngbaggen av information från olika riktningssignaler, såsom solens position eller himmelns polarisationsmönster (ett ljusmönster skapat från spridningen av solljus). Dessa integreras alla i dyngbaggens *interna kompass*.

Som dyngbaggforskare händer det ofta att man finner sig stirrandes på en dynghög mitt på savannen. Efter att själv ha gjort detta ett par gånger började jag så småningom fundera på hur storleken på dyngbaggen påverkar dess förmåga att hålla en rak kurs. När en bagge rör sig framåt, skapas mekaniska störningar i lederna, vilka kan bidra till fluktuationer i dess rörelse framåt, vilket i sin tur kan leda till att dyngbaggen avviker från sin kurs. Hur påverkar då steglängden, som är kortare hos de mindre arterna, skalbaggens förmåga att rulla rakt? I **Artikel I**, tittade jag närmare på denna fråga genom att jämföra *orienteringsstrategin* hos två närbesläktade dyngbaggearter av olika storlek. Jag fann att störningen som genereras över ett visst avstånd är omvänt proportionell mot steglängden. Detta betyder att över samma avstånd kommer mindre baggar, som tar fler steg än större individer, ha en mer slingrig rullsträcka. Intressant nog, i deras naturliga miljö tar båda dyngbaggar ungefär lika många steg innan de gräver ner sin boll, men på grund av störningen som genereras, kommer mindre baggar hamna radiellt närmare dynghögen än större baggar.

För de flesta savann-levande dyngbaggar är solen en dominant riktingsgivare. Men gäller detta även för dyngbaggar som vill styra rakt genom miljöer med tätare vegetation? I **Artikel II** fokuserade jag på orienteringsstrategin hos en dyngbaggeart som lever i miljöer där solen ofta är skyddad av moln eller trädtoppar och fann att dessa dyngbaggar främst förlitar sig på himmelns polarisationsmönster för att styra rakt. Detta tyder på att den visuella miljön inom vilken arten är aktiv kan spela en roll för hur olika riktingsgivare vägs samman för orientering. Att systemen är mer komplexa än så blev tydligt då jag utökade mina jämförande studier till tre olika släkter av dyngbaggar som lever inom samma miljö. I **Artikel III** fann jag att varje släkt av de tre

jag testade, hade sin egen strategi: en förlitade sig främst på solen för att styra sin väg, en annan förlitade sig på polarisationsmönstret och en tredje tycktes inte främst förlita sig på någon av dessa två riktningsgivare. Inte oväntat spelar även dyngbaggens fylogeni en viktig roll för dess orienterings-strategi.

Det är viktigt att notera att en dyngbagge som förlitar sig främst på solen, kommer inte plötsligt att rulla i cirklar så snart denna riktnings-signal försvinner bakom ett moln eller om skalbaggen rullar in under skuggan av ett träd. Istället förlitar sig dyngbaggen på sin näst mest populära riktningsgivare: himmelns polarisationsmönster. I **Artikel IV** byggde jag en uppställning där jag introducerade en simulerad sol tillsammans med ett simulerat polarisationsmönster. Genom att ändra egenskaperna av dessa två riktnings-signaler (till exempel genom att ändra den relativa ljusintensiteten) och analysera dyngbaggens respons, fann jag att ju mer opålitlig en riktnings-signal är, desto mindre vikt läggs på denna signal i dyngbaggens kompass. Detta avslöjar en av de grundläggande principerna bakom dyngbaggekompassens förmåga att anpassa sig till olika visuella miljöer.

Jag hoppas att mina fyra artiklar bidragit till en större förståelse för vilka utmaningar en styrande insekt stöter på och hur den löser dessa. Oavsett väder, terräng eller stirrande forskare, kommer dyngbaggen att fortsätta sin färd framåt.





Paper overview

This thesis is primarily based on my four main studies regarding the heading direction network and straight-line orientation behaviour of South African ball-rolling dung beetles. However, where relevant, I will also refer to the five additional studies that I have co-authored. These five papers will be denoted with an asterisk (*) when appearing in the text. Below, I list the principal question addressed in each of my four main studies with a brief summary of what was achieved. Throughout the thesis, I will refer to these four papers by their roman numerals as given below.

Paper I

Khaldy L, Peleg O, Tocco C, Mahadevan L, Byrne M, Dacke M. (2019). The effect of step size on straight-line orientation. *J R Soc Interface* **16**, 20190181.
<https://doi.org/10.1098/rsif.2019.0181>

What influence does the step size of the agent have on its's ability to maintain a straight bearing? What is the weight relationship of internal and external compass cues in the heading direction network of the dung beetle?

If an animal relies exclusively on internal sensory information while travelling along a trajectory, the directional error that is generated with each ensuing step will accumulate and effectively cause the animal to spiral. Only when the animal is allowed to use directional information from external compass cues can it correct for errors in its bearing. In this study, I investigated the effect of directional error on straight-line orientation in two closely related, but differently sized, species of dung beetles; *Scarabaeus ambiguus* Boheman and *Kheper lamarcki* (Mac Leay) [Scarabaeini]. For each species, I characterised the size of the directional error generated with each step, in the presence and absence of external compass cues, and investigated the influence of this error on the tortuosity of the travelled path. Next, we modelled the weight given to external compass cues over internal proprioceptive cues in the heading direction

network of the beetle. From our results we concluded that the directional error that unavoidably accumulates as the beetle travels, is relative to the step size of the animal and that both species weight the two sources of directional information in a similar fashion. Furthermore, and perhaps not surprisingly, the dung beetles attribute significantly greater weight to external directional cues over internal directional information while performing straight-line orientation.

Paper II

Khaldy L, Tocco C, Byrne M, Baird E, Dacke M. (2019). Straight-line orientation in the woodland-living beetle *Sisyphus fasciculatus*. *J Comp Physiol A* **206**, 327-335.
<https://doi.org/10.1007/s00359-019-01331-7>

Are all ball-rolling dung beetle species guided by a common weighting of directional cue information in their heading direction network?

Prior to this study, nearly all behavioural work regarding straight-line orientation in dung beetles had been performed on ball-rolling dung beetle species present in vast, open habitats, and had concluded that the sun is given the greatest relative weight in the heading direction network. Here, I investigated straight-line orientation in the South African woodland-living beetle *Sisyphus fasciculatus* Boheman [Sisyphini], present in habitats with densely packed trees and tall grass. I concluded that, contrary to all previous observations on diurnal ball-rolling beetles, *S. fasciculatus* relies predominantly on directional information from the celestial pattern of polarised light.

Paper III

Khaldy L, Tocco C, Byrne M, Dacke M. (2021). Compass cue integration and its relation to the visual ecology of three tribes of ball-rolling dung beetles. *Insects* **12**, 526. <https://doi.org/10.3390/insects12060526>

What role does ecological niche and/or tribe play in the weighting of directional cue information in the heading direction network of the beetle?

In this study, I continued to explore the relative weighting of directional information in three species of ball-rolling South African dung beetles, from three different tribes living within the same savanna biome, but in different habitat types. I found that species within a tribe share the same orientation strategy, but that this strategy differs across tribes. Inter-tribal differences in body size, eye size, and overall morphology, most likely influence how species within each tribe weight the sources of directional information available to them. Nevertheless, dung beetles manage to solve the challenge of straight-line orientation via a weighted combination of visual cues that are particular to the habitat in which they are found. However, this system is dynamic, allowing the beetles to operate equally well, even in the absence of the cue they typically assign the greatest relative weight.

Paper IV

Khalid L, Foster J, Yilmaz A, Belušič G, Gagnon Y, Tocco C, Byrne M, Dacke M.
The interplay of directional information provided by unpolarised and polarised light in the heading direction network of *Kheper lamarcki* (*Manuscript submitted*)

How does the relative reliability of different directional cues influence the weight relationship in the heading direction network of the beetle?

The sun is the most prominent directional compass cue in the heading direction network of the diurnal ball-rolling dung beetle *Kheper lamarcki*. If this celestial body is occluded from the beetle's field of view, which can occur by passing clouds or when rolling in the shade of a tree, the distribution of the relative weight between the directional cues that remain shifts in favour of the celestial pattern of polarised light. In this lab-based study, I investigated the weight relationship of directional information from the sun (simulated by a green LED) and the celestial polarisation pattern (simulated by an overhead band of polarisation) in the heading direction network of the beetle. By altering the intensity, degree and direction of polarisation of the overhead light, this allowed me to determine how the weight relationship of the two sources of light is influenced by their relative reliability. From my results, I can conclude that the heading direction network of *K. lamarcki* relies on directional information in a Bayesian manner; directional information conveying the highest certainty at any moment in time is afforded the greatest weight in the heading direction network of the animal.



Background

The purpose of my work

To travel along a given direction, towards or away from a fixed point in space, oftentimes requires the possession of a great navigational toolkit; *a biological compass* or *a heading indicator* (see *Box 1*). To maintain a desired heading, the navigator (or more accurately its compass) must be able to sift through and extract relevant directional information from the vast range of external cues presented. As it is moving, directional information generated by the navigator itself, such as body rotations or leg movements, might also be considered and integrated into the compass. This means that, to maintain a desired direction, the compass must not only be able to extract the correct directional information, but also continuously compare the current heading to the desired one and reorient the navigator in reference to the stable cues provided. Although an extensive number of studies within insect navigation have provided excellent insight into the directional information utilised by and integrated into the heading direction networks of insects, understanding exactly how insect are able to steer with respect to multiple orientation cues, remains to be answered. It is here my thesis begins.

Because of its relatively straightforward orientation behaviour, the ball-rolling dung beetle provides an excellent model in which to study *the heading direction network* and the factors by which it is influenced. Found on all continents of the globe (except for the Antarctic), differing in shape, colour and size, these animals have one distinct behaviour in common; the ability to gather and shape a piece of dung into a ball and roll it away from the dung pat in as straight of a trajectory as the terrain allows (**Paper I**; **Paper II**; **Paper III**; Baird et al., 2010; Byrne et al., 2003; Dacke et al., 2013a; Dacke et al., 2013b; Dacke et al., 2014; Dacke et al., 2021).

By investigating how *size* (**Paper I**), *ecological niche* and *phylogeny* (**Paper II** and **Paper III**) and *visual conditions* (**Paper IV**) influence the straight-line orientation behaviour of these insects, I explore the challenges faced, and the solutions presented by their heading direction networks. With my work, I hope to provide an insight to the dynamic nature of the biological compass and its ability to change and adapt to different visual environments.

The ball-rolling dung beetle

With over 6000 species (Cambefort and Hanski, 1991*b*) dung beetles represent a substantial range and variety in morphological and ecological niche. Common for most is their affinity for dung, however the way it is consumed varies. In principle, dung beetles can be categorized into three functional types: *endocoprids* (dwellers), *paracoprids* (tunnelers) and *telecoprids* (rollers).

While *endocoprids* feed directly on the dung pat, *paracoprids* form tunnels underneath the pile, disappearing with a piece of dung to their chambers where they consume it in peace. For *telecoprids*, encompassing nearly 600 species of dung beetles, the interaction with dung can stretch several tens of meters away from the dung pile (**Paper I**). These beetles shape a piece of dung into a ball which they roll away from the pat. This behaviour is believed to be derived as a means of escaping the fierce competition for dung at the pat (Cambefort and Hanski, 1991*a*). After around 6 minutes of rolling (Dacke et al., 2019), the ball-rolling dung beetle will burrow into the ground with its ball. Once the beetle has consumed the dung, it emerges from the ground, commences the quest for food and starts the cycle all over again.



Box 1. Navigation and Orientation

Travelling insects can guide their forward route using one of two primary strategies:

Navigation. Navigation requires the use of a compass that informs the animal of its direction in relation to a set reference point, no matter where the animal is in space. Navigation can, in principle, be categorized into two classes: *long-distance migration* and *homing*.

Long-distant migrants, such as Bogong moths, navigate to the Alpine caves using a magnetic compass in conjunction with landmarks (Dreyer et al., 2018) and monarch butterflies reach their overwintering grounds in Mexico by the use of their time-compensated sun compass (Perez et al., 1997).

Homing by path-integration also requires an odometer. Ants and bees (Collett, 1996) continuously keep track of the distance travelled (by their odometer) and their global direction (by their compass) in relation to a select goal (their nest or food source). This information is then integrated to produce a single 'home vector' that takes them directly back to their point of origin.

Orientation. An orienting animal has the aim to travel along a given bearing, but does not necessarily have a select goal. The ball-rolling beetle is an animal that orients; once it has formed its ball of dung, it chooses a seemingly arbitrary heading direction (Baird et al., 2010) and continuously integrates sensory cue information to steer its trajectory straight across the sandy terrain. Essentially, contrary to navigation, the only requirement of the guidance system of an animal that orients is to hold a constant direction in reference to the directional cue.

(continues on next page)

Compass or heading indicator?

There is a notion that animals using directional cue information only for steering - like the ball-rolling dung beetles - do not truly rely on a compass, but instead possess a *heading indicator* (Guilford and Taylor, 2014). A heading indicator will not compensate for the apparent change in position of the external reference cues that are integrated, such as the apparent movement of the sun across the sky over the day. Therefore, simply travelling in constant bearing to this celestial reference cue would steer the animal in close to opposite directions in the morning and in the afternoon. For short term movements, such as the ball-rolling journey of the beetle (Dacke et al., 2019), this is however not a problem. What constitutes as a 'heading indicator' versus a 'compass' is still fairly vague, and has yet to be fully accepted in insect navigation literature. For this reason, this distinction is not made in this thesis. It is, nonetheless, important to note to the reader, that in the context of dung beetle orientation throughout this thesis, the term biological compass and heading indicator always refers to the orientation mechanism involving the integration of directional cue information from appropriate sensory signals to steer along a given bearing.

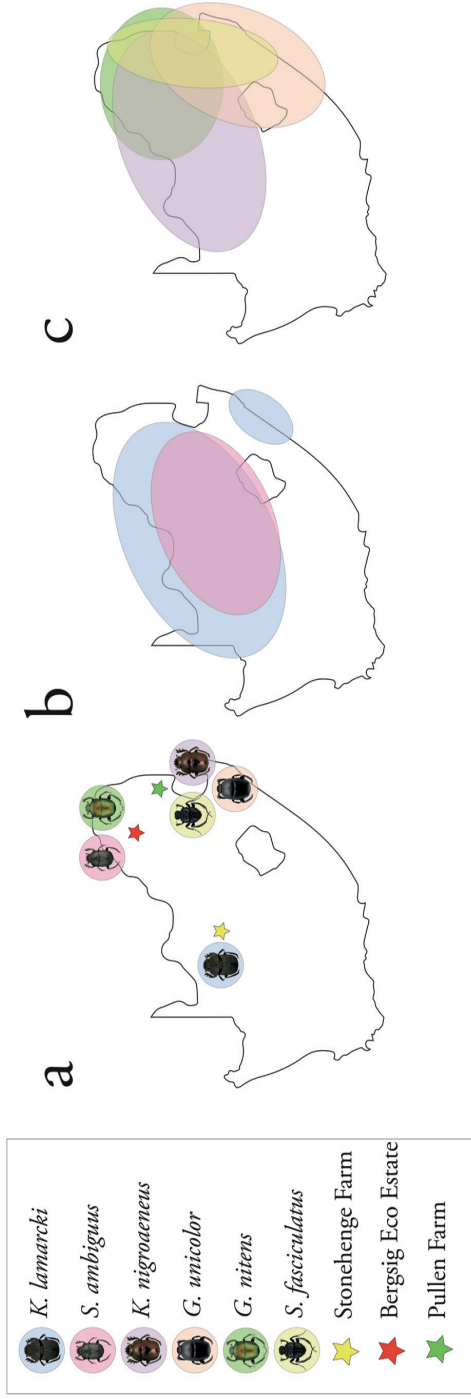
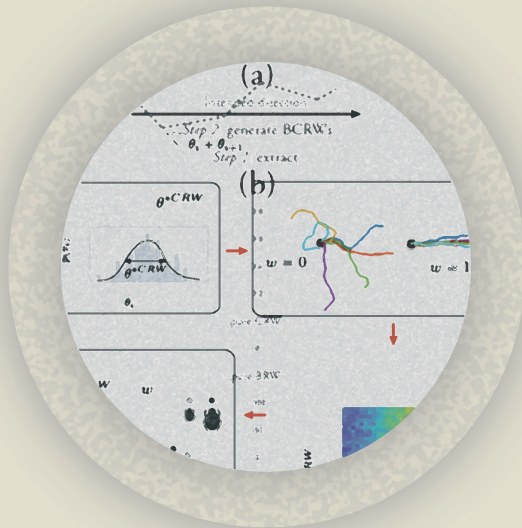


Figure 1. Map of field sites and distribution of species. The behavioural studies presented in this thesis focus on six species of South African ball-rolling dung beetles (*Kheper lamarcki* (Paper I; II; IV); *Scarabaeus ambiguus* (Paper I); *Kheper nigroaeneus* (Paper III); *Garreta unicolor* (Paper III); *Garreta nitens* (Paper III); *Sisyphus fasciculatus* (Paper II; III)), collected at three different field sites in South Africa (a). A rough estimate of the species distributions across South Africa is shown in b and c (data modified from Scholtz and Ramuashu 2021).



The influence of noise in a biological compass system

As the dung beetle rolls across the sandy terrain of the South African savanna, noise unavoidably accumulates in the beetle's motor and sensory system (Rung, 2007). Noise caused from the integration of internal cues generates **motor error**, where the animal's perceived joint position does not match its true joint position, and noise caused from the integration of external cues leads to **compass error**, where the perceived position of a cue does not quite match its true position, consequently affecting the motor output of its straight-line orientation behaviour with each ensuing step.

The ball-rolling dung beetle provides an excellent model to study the influence of noise

Previously, the detailed influence of noise on the ability to maintain a straight course had only been studied mathematically (Cheung et al., 2007). As the primary goal of ball-rolling beetles is to maintain a straight bearing while moving forward (**Paper II**; **Paper III**; Dacke et al., 2021), they offer an excellent model species with which to tackle this question from a behavioural point of view. With a wide array of species, ranging from a few millimetres to a few centimetres (Cambefort and Hanski, 1991*b*), this diverse group of insects can help understand how noise affects the biological compass in the absence and presence of external directional reference cues. By studying the effect of motor and compass error on straight-line orientation in two differently sized, but closely related, ball-rolling beetles; *Scarabaeus ambiguus* (pronotum width of 1 cm, body length of 1.5 cm and step size of 1.6 cm) and *Kheper lamarcki* (pronotum width of 2 cm, body length of 3 cm and step size of 2.6 cm) [Scarabaeini] (*Figure 2*), I set out, in **Paper I**, to investigate *i*) how the error associated with each step of the beetle (*step size error*) influences its straight-line orientation behaviour, in the absence and presence of external cues and *ii*) how external and internal directional cue information (self-generated motion signals) is weighted in its heading direction network. I behaviourally estimated the motor error generated per step in both species and used this as an input

parameter into a *biased correlated random walk* (BRCW) model (Bailey et al., 2018), developed together with researchers from Harvard University. From the BRCW model, the compass error could be estimated (see *Box 2*) and the weight given to external visual cues over internal proprioceptive cues could be determined.

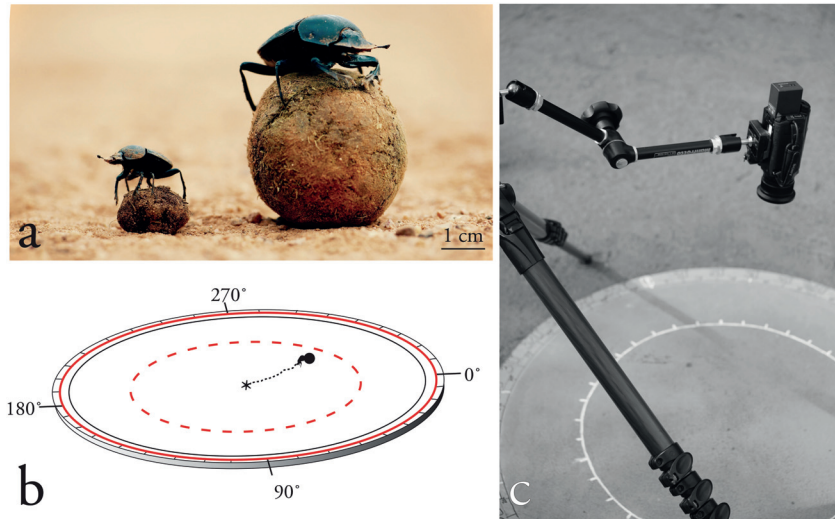


Figure 2. Description of the experimental design (Paper I). Individuals of *Scarabaeus ambiguus* (left) and *Kheper lamarcki* (right) are depicted side-by-side for size comparison (a). Photo: Christopher Collingridge. For all treatments, a beetle was placed alongside a ball in the centre of a circular, sand-coated arena (b) and filmed with an overhead camera (c). The beetle was allowed to roll its ball to the perimeter of the arena, where the exit angle was noted. Three differently sized arenas were used depending on the species tested (b): 50 cm (*S. ambiguus* and *K. lamarcki*, black solid line), 33 cm (*S. ambiguus*, red dotted inner circle) and 52 cm (*K. lamarcki*, red solid outer circle) radius.

The role of the sun in straight-line orientation of Scarabaeus ambiguus

To be able to roll along a straight trajectory, the dung beetle, and travelling insects in general, must integrate relevant directional information from appropriate sensory cues into their heading direction network (see *Box 1*). Usually, these cues are derived from two main sources of directional information; internal mechanosensory cues, such as body rotations or leg movements (Bisch-Knaden and Wehner, 2001; Wittlinger et al., 2006) and external reference cues such as sky compass cues (Paper I-IV; Byrne et al., 2003; Dacke et al., 2014), terrestrial cues (Cartwright and Collett, 1982; Fukushi and Wehner, 2004) or magnetic cues (Dommer et al., 2008; Fleischmann et al., 2020; Guerra et al., 2014).

Past studies on the large ball-rolling dung beetle, *K. lamarcki*, have undoubtedly demonstrated that in its heading direction network, the directional information provided by the sun is afforded the greatest weight when supporting straight-line

orientation (see *Compass cues used by the ball-rolling dung beetle*). However, the role of the sun in the heading direction network of the smaller, closely related, ball-roller, *S. ambiguus*, was up until **Paper I** unknown. Therefore, in this study, I first demonstrated the role of the sun in the heading direction network of *S. ambiguus*. This was done by allowing individuals of this species to roll under an open, clear sky in the presence of a mirrored sun, while simultaneously shading the real sun from the beetle's field of view.

When the apparent position of the sun was changed by 180° with the use of a mirror, *S. ambiguus* responded to this azimuthal change of apparent sun position with the same order of magnitude as its larger cousin, changing its bearing direction by 150° (*K. lamarcki* changed its bearing direction by 140° when presented with the same experimental paradigm). My findings demonstrate that the heading direction network of *S. ambiguus* integrates directional information from the sun to orient, and suggests that, much like its larger cousin, the heading indicator of this beetle predominantly relies on directional information from the sun during straight-line orientation.

Box 2. Biased Random Walk and Correlated Random Walk

Two main random walk models are used to describe how an agent navigates through its environment:

Biased random walk. An agent moving forward, guided by an external cue, is moving by means of a biased random walk (BRW) (Hill and Häder, 1997). Here, the agent will move in a given direction in relation to an external directional cue.

Correlated random walk. If external cues are absent, the animal instead moves by means of a correlated random walk (CRW) (Bovet and Benhamou, 1988; Kareiva and Shigesada, 1983). Here, the agent relies on internal cues and each step is intended to point in the same direction as the previous.

The effect of noise on straight-line orientation

Based on my first findings in **Paper I**, a similar celestial orientation strategy for both the smaller, *S. ambiguus* and the larger *K. lamarcki* could be identified, but the question of the influence of size (or more specifically step length) emerged: if noise is generated by each ensuing step, how does the *step size* of a beetle influence its ability to maintain a straight bearing during straight-line orientation?

I first explored this question from a purely behavioural standpoint. This was done by defining the orientation precision of the beetles' straight-line trajectories when rolling across flat, sanded arenas of different sizes, 20 consecutive times. One arena had a radius of 50 cm, and another two had radii of 32 cm and 52 cm -equivalent to 20 step-lengths of *S. ambiguus* and *K. lamarcki* respectively (*Figure 2b*). In the context of straight-line orientation, orientation precision can be determined from the mean vector length, R , of 10 or more consecutive rolls (Dacke et al., 2019; Foster et al., 2019*; Foster et al., 2021*), where a value of 0 indicates a random distribution of angles (where data is not clearly bimodal), and a value of 1 indicates no dispersion in distribution of angles (*Figure 3*).

From the angular spread in bearing direction over 20 consecutive rolls performed by each species in each paradigm (across a radial distance of 20 steps or 50 cm), it became evident –as expected– that, even under an open sky, with several external directional cues available, the headings travelled by the beetles are subject to noise. In addition, when moving over the same distance, this noise appears to be inversely proportional to the step size of the beetle: when individuals of the smaller *S. ambiguus* and the larger *K. lamarcki* were allowed to roll across a radial distance of 50 cm, the smaller beetle had a significantly shorter mean vector length compared to that of its larger cousin. However, when instead allowed to roll over a radial distance equal to 20 steps, no significant difference in mean vector length was found between the two ball-rollers. The results demonstrate that, over the same absolute distance, the smaller beetle is less able to maintain a straight bearing when rolling under an open sky.

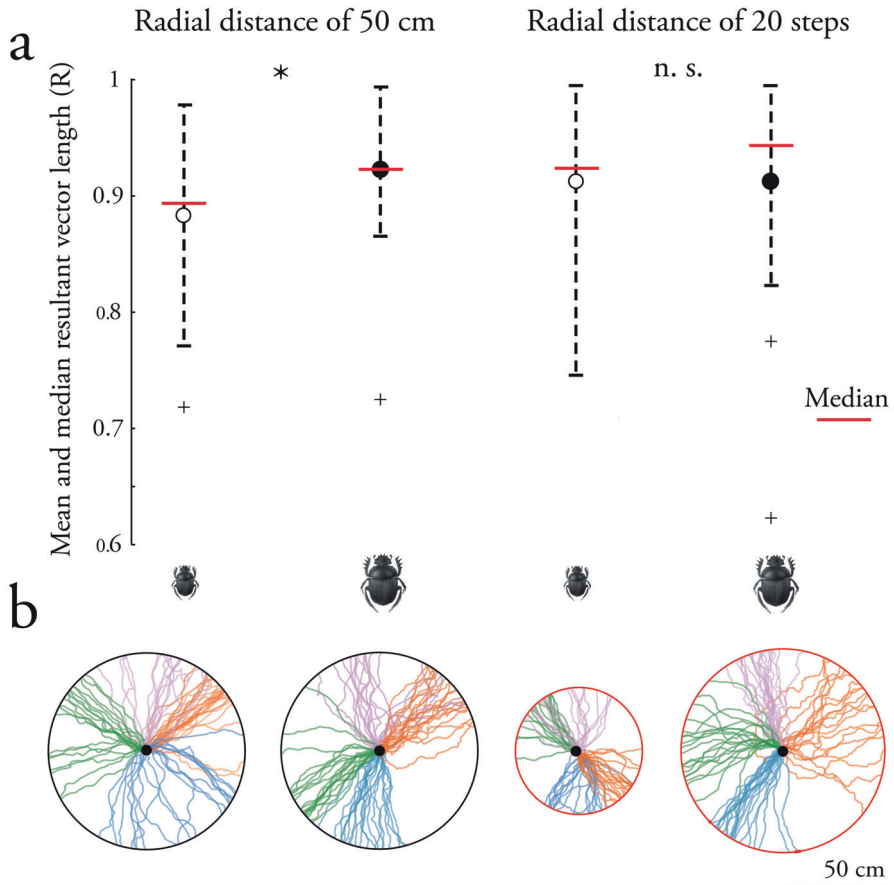


Figure 3. Orientation performance in the presence of external visual cues (Paper I). As a measure of orientation performance (a), the mean vector length for each beetle was calculated from 20 trajectories over a radius equivalent of 50 cm, as well as of a radius equivalent of 20 step lengths of the corresponding species (32 cm for *Scarabaeus ambiguus* and 52 cm for *Kheper lamarcki*) (white circle: mean value for *S. ambiguus*; black circle: mean value for *K. lamarcki*; red solid line: median value for *S. ambiguus* and *K. lamarcki*). An R-value of 1 indicates that the beetles maintained the same direction over 20 rolls. When rolling over a radius of 50 cm, the smaller species, *S. ambiguus*, showed a significantly shorter resultant vector length compared to its larger cousin (R(*S. ambiguus*): 0.88 ± 0.02 ; R(*K. lamarcki*): 0.92 ± 0.01 , $p < 0.1$, $N = 20$). However, no significant difference was seen when both species rolled over a distance equivalent to 20 steps (R (*S. ambiguus*): 0.91 ± 0.015 ; R (*K. lamarcki*): 0.91 ± 0.02 , $p = 0.42$, $N = 20$). Paths travelled by four individuals for each species are shown in b (from left: *S. ambiguus* (50 cm); *K. lamarcki* (50 cm); *S. ambiguus* (32 cm); *K. lamarcki* (52 cm)). Each colour represents 20 trajectories of one individual. * = $p < 0.05$; n. s. = $p > 0.05$.

The effect of the greater accumulation of noise in smaller versus bigger species of dung beetles can be observed when tracking beetles in their natural habitat: when allowed to roll from the same dung pile in nature (a likely occurrence for these beetles, as they are observed to actively forage within the same habitat (see *Figure 1*)), the total path length to the final burial spot did not differ between the smaller *S. ambiguus* and the larger *K. lamarcki*. Yet, if measured radially, the smaller sized beetles clearly appeared to bury their dung balls at a significantly shorter radial distance from the pile (*Figure 4b*). The correlation between the distance an insect travels and its body size can also be observed in bees and darkling beetles: here, similar to what has been observed in the dung beetles, larger conspecifics tend to forage further than smaller ones (Crist et al., 1992; Greenleaf et al., 2007). A possible explanation for these dispersal differences can be that the accumulation of noise in the compass system of these insects is, much like for the beetles, proportional to the size of the animal. This same size-related phenomenon can also be observed in ants, where over an absolute distance, smaller sized individuals travel more tortuous paths than their larger conspecifics (Palavalli-Nettimi and Narendra, 2018). Interestingly, the results observed in nature suggests that the heading indicator of the beetle does not compensate for the directional challenges faced by the smaller beetles rolling across the same terrain as their bigger relatives and competitors; the noise is inversely proportional to the step size of the beetle. However, the proportion of the noise allotted to motor error versus compass error could not be analysed from the behavioural data alone. For this, a mathematical model was implemented (*Box 3*) with the help of my collaborators at Harvard University, MA, USA.

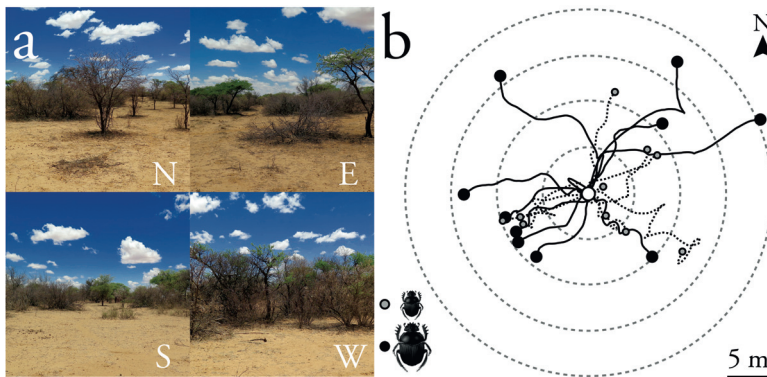
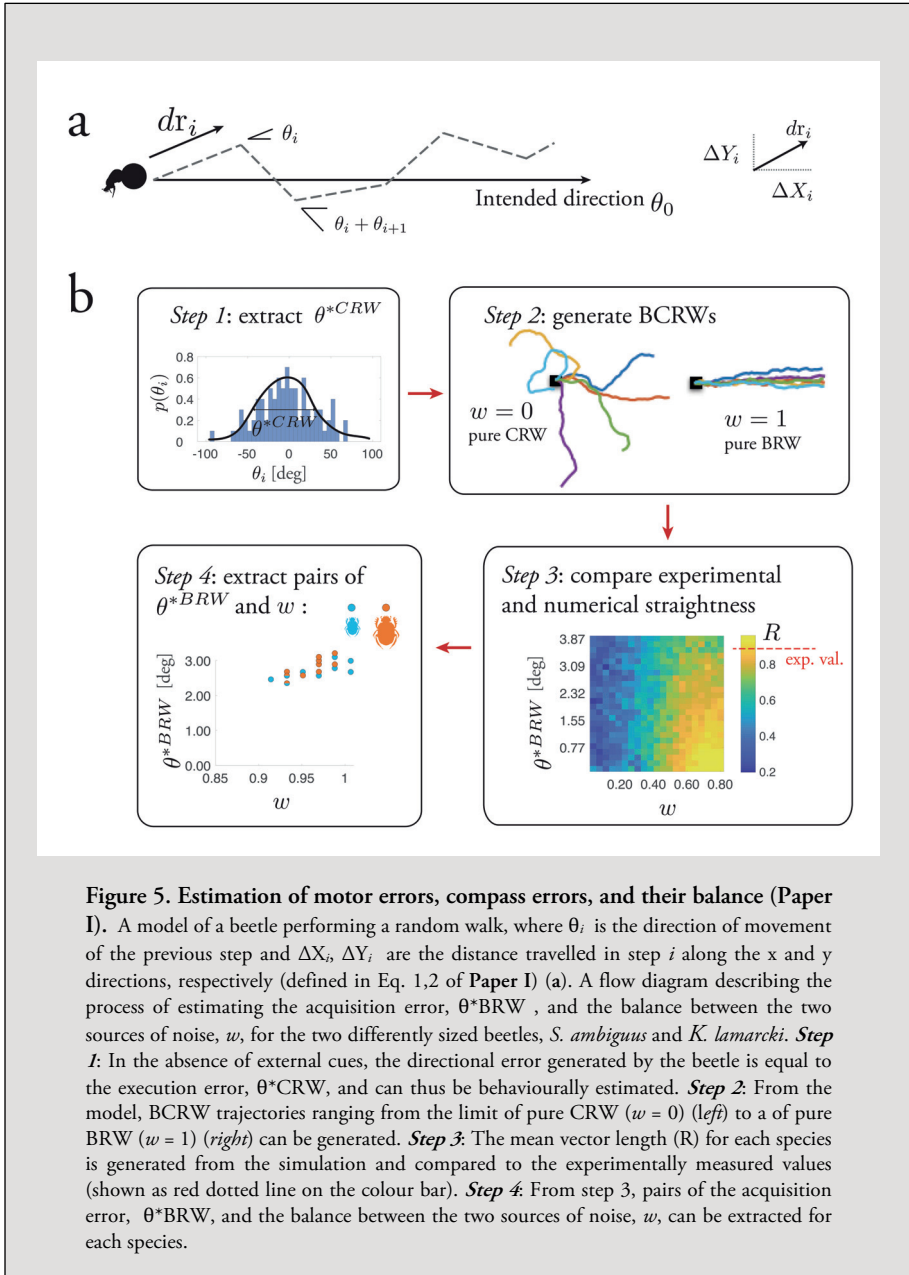


Figure 4. Rolling trajectories of *Scarabaeus ambiguus* and *Kheper lamarcki* in natural terrain (Paper I). The smaller *S. ambiguus* and the larger *K. lamarcki* were allowed to form a dung ball and roll it away from a dung pat in their natural environment (*N*: north; *E*: east; *S*: south; *W*: west.) (a). Their trajectories (*dashed black line*: *S. ambiguus*; *solid black line*: *K. lamarcki*), were recorded until they started to bury their balls (*grey circles*: *S. ambiguus*; *black circles*: *K. lamarcki*) (b). Compared to the larger *K. lamarcki*, *S. ambiguus* rolled a significantly shorter radial distance from the pat before burying its ball (*S. ambiguus*; $7.56 \text{ m} \pm 1.05 \text{ m}$, *K. lamarcki*; $12.45 \text{ m} \pm 1.28 \text{ m}$, $N = 10$) ($p < 0.01$, Wilcoxon Rank Sum).

Box 3. Compass error can be estimated using a biased correlated random walk model

A biased correlated random walk model (Bailey et al., 2018) was used to estimate the compass error and determine the relative weighting of internal and external cues in the heading direction network of the beetle. The behaviourally extracted values given for the motor errors for each species, were implemented in the model as input parameters (*Figure 5, step 1*). From here, trajectory examples were extrapolated, ranging from conditions when the agent is only reliant on internal cues (a pure CRW), to when the agent is only reliant on external cues (a pure BRW) (*Figure 5, step 2*). From these simulations, mathematically generated mean vector length (R) values were created that were in turn compared to the experimentally obtained mean vector length values attained from rolling the beetles under an open sky (*Figure 5, step 3*). When fitting the experimentally obtained R-values with the modelled ones, compass errors of 1.16° (*S. ambiguus*) and 1.31° (*K. lamarcki*) could be extracted. Furthermore, the balance between compass errors and motor errors (termed w , where the limit of a pure CRW is $w = 0$, and a limit of pure BRW is $w = 1$) was estimated by the model and determined to $w = 0.84$ for *S. ambiguus* and $w = 0.83$ for *K. lamarcki*. Interestingly, like the motor errors, the compass errors and the balance between these two sources of errors did not differ for the two species (*Figure 5, step 4*). This indicates that the compass system of the differently sized beetles is not designed to compensate for the faster accumulation of errors generated by the smaller navigator as it exits from the dung pat.

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Determining motor and compass error

When integrating directional information from both internal and external cues to steer straight under a clear sky, the directed movements of the dung beetle could best be represented by the means of a *biased and correlated random walk* (BCRW). According to this model, the noise accumulated in each step is generated from the integration of internal as well as external directional cues. In order to separate these two sources of noise, we needed to first extract the noise generated by internal cues exclusively. In the absence of other cues, motor output is governed solely by proprioceptive cues. For a beetle rolling in complete darkness, the angular error generated by each step can therefore acts as a proxy for motor error.

Motor error is determined in the absence of external directional cues

Any agent moving forward relying on internal mechanosensory information alone, will not succeed in travelling any greater distance from its initial location. This is because each subsequent step taken by the agent will deviate slightly from the former direction, ultimately causing the agent to stray from its intended route (Cheung et al., 2007; Cheung et al., 2008). Despite this predicament, there are animals that rely solely on internal proprioceptive cues for directed movements. These include hunting spiders, that find their way back to the food site using internal information from their lyriform slit-sense organs alone (Barth and Seyfarth, 1971), or cockroach larvae, that navigate back to their shelter using only kinaesthetic cues (Durier and Rivault, 1999). However, in these situations, the distances travelled are comparably short, limiting the accumulation of mechanosensory noise. Animals that travel relatively far, like the dung beetles (around 10-20 m, see *Figure 4b*), must instead use external compass cues in combination with internal cues to successfully orient or navigate (Cheung et al., 2007; Collett, 1996; Dacke et al., 2020; Heinze et al., 2018; Kim and Dickinson, 2017; Srinivasan et al., 1996).

To determine the motor error generated by each beetle species, individuals were allowed to roll in complete darkness. Interestingly, when rolling devoid of external visual cues, the trajectories of each species differed in straightness (Batschelet, 1981) over a radial distance of equal absolute length (50 cm), but not equal relative length (20 steps of the species) (*Figure 6*). These results are similar to those found under the open sky, indicating that the noise generated per step is the same in the two beetle species. From the trajectories of both species, angular error per step was calculated as the absolute difference in bearing direction between two consecutive foreleg-surface contacts, and from this, motor errors of 33° for *S. ambiguus* and 29° for *K. lamarcki* could be determined.

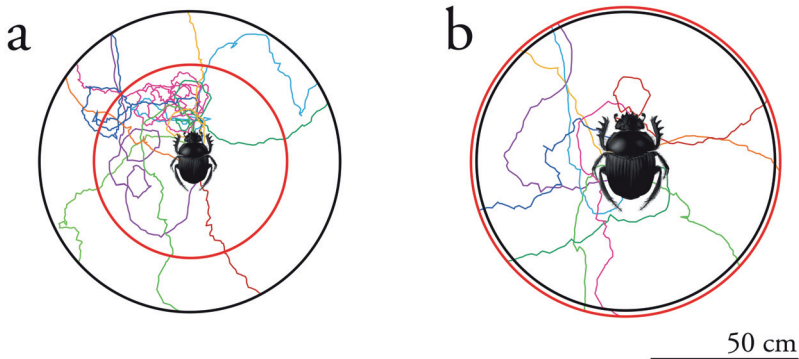


Figure 6. Rolling trajectories in the absence of visual cues (Paper I). When allowed to roll a dung ball from the centre of a flat, sand-coated arena, in complete darkness, *Scarabaeus ambiguus* (a) obtained a significantly higher tortuosity than *Kheper lamarcki* (b) over a radial distance of equal absolute length (50 cm radius, *black perimeter*). Over a radial distance corresponding to 20 steps for each species respectively (*red perimeter*), no significant difference in tortuosity was recorded between the species.

Compass error is estimated through a biased and correlated random walk model

Once the amount of motor error was estimated, this could be included in our model and used to estimate the noise generated by the integration of external compass cues into the heading direction network (compass error). This then allowed us to estimate the weight given to internal and external cues in the heading direction network of the beetle as it rolls across the savanna.

From the trajectories of the beetles under the open sky, it is apparent that there is some noise present in the beetle's heading indicator when rolling outside. However, due to their relatively straight trajectories across the flat, sanded arena (R value over a radius of 20 steps: R (*S. ambiguus*) = 0.9; R (*K. lamarcki*) = 0.9), it is clear that this noise is smaller than what is generated in the dark (compare *Figure 3* and *Figure 6*). Not surprisingly, from our model (see *Box 3*) the compass error of 1.16° per step for *S. ambiguus* and 1.31° per step for *K. lamarcki* was significantly less than the motor error found for each species (33° for *S. ambiguus* and 29° for *K. lamarcki*). What is interesting is that the noise generated by the integration of internal cues as well as external cues is inversely proportional to the step size of the beetle. This indicates that the heading direction network of the smaller beetle is just as precise as that of the larger, further highlighting that the heading direction network of the smaller beetle is not evolved to compensate for the directional challenges that arise due to differences in stride length. This can be an energetically expensive disadvantage for an orienting insect that aims to travel the same distance irrespective of size, but as my study suggests (*Figure 4b*) burying

at different distances from the dung pile might be an advantage, as it decreases the chances of beetles ending up burying in the same spot, thus limiting the opportunity of competitors to steal another beetle's ball.

In addition to estimating the compass error, our model also estimated the relative weight of internal proprioceptive cues and external reference cues under the open sky. It was found that this relative weight in the heading indicator of the beetle, when rolling outside, was significantly shifted to external reference cues, allotting approximately 85% of the directional weight in the heading direction network to external cues. This applied to both species, and stand in line with our previous observations of ball-rolling dung beetles and their dependence on celestial cue input for straight-line orientation (Dacke et al., 2013b; Dacke et al., 2019; Foster et al., 2021*; el Jundi et al., 2015a*).



Compass cues used by the ball-rolling dung beetle

As it makes its way around small bushes and tufts of grass, a savanna-living ball-roller –or rather, its brain– integrates relevant directional information from several different compass cues. To maintain its bearing, the brain continuously compares the desired heading with the current one, adjusting for any deviations in the beetle’s path, until a suitable place to bury and consume its ball is found. The end of the ball-rolling adventure is very likely determined by the terrain (Osberg et al., 1993; Osberg et al., 1994), as well as the size of the ball-roller (for more see *The influence of noise in a biological compass system*). In general, the directional information used to guide animals throughout their journey, depends on the availability of cues and the navigator’s ability to detect them. Here, I describe the most prevalent compass cues used by ball-rolling dung beetles (and other travelling insects).

The sun

The sun plays a dominant role in the heading direction network of many diurnal ball-rolling dung beetles

The sun compass in honeybees was discovered over 60 years ago (Frisch and Lindauer, 1956), clearly demonstrating that honeybees use directional information from the sun to navigate to their food source. Ever since then, a vast range of arthropods have been confirmed to utilise directional information from the sun to guide their navigational tasks: monarch butterflies and other migratory butterflies use this information to guide their routes over long distances (Merlin et al., 2009; Mouritsen and Frost, 2002; Nesbit et al., 2009; Perez et al., 1997), sandhoppers reference this celestial body to get themselves to and from the shore (Forward et al. 2009; Scapini, Fallaci and Mezzetti 1996; Williamson 1951), desert ants integrate directional information from the sun to

navigate back to the nest (Lebhardt and Ronacher 2015; Muller and Wehner 2006) and dung beetles use it to steer straight across the savanna (**Paper I**; **Paper III**; Byrne et al., 2003; Dacke et al., 2014; Dacke et al., 2019; el Jundi et al., 2015a*; Smolka et al., 2016).

For most diurnal ball-rolling dung beetles studied in the context of straight-line orientation, directional information from the sun receives the greatest relative weight in their heading direction network (**Paper I**; **Paper II**; Byrne et al., 2003; Dacke et al., 2014; Dacke et al., 2019; el Jundi et al., 2015a*). This is demonstrated by using the simple, yet powerful method of reflecting the apparent position of the sun with a mirror, while simultaneously obstructing the real sun from the animal's field of view. Responding to the positional change of the apparent sun, while the position of all other celestial cues, such as the celestial polarised light pattern (Horváth et al., 2014; Pomozi et al., 2001; Suhai and Horváth, 2004) as well as the intensity (Warrant et al., 2020) and colour gradient of the sky (Coemans et al., 1994) remain unchanged, is a clear indication of the relatively high weight given to the directional information provided by the sun in the heading direction network of the animal.

Apart from studies in dung beetles, this classic 'mirrored sun' method has also been used in various other studies of arthropods, such as ants (Wystrach et al., 2014), sandhoppers (Pardi and Papi, 1953) and marine isopods (Ugolini and G, 1988). For the dung beetle, a dominant use of the sun as a directional cue is not only demonstrated for *Kheper lamarcki* (Dacke et al., 2014; Dacke et al., 2019; el Jundi et al., 2015a*), but also for *Pachysoma femoralis* (Byrne et al., 2003), *Scarabaeus ambiguus* (**Paper I**) and *K. nigroaeneus* (**Paper III**), all present in similar visual environments.

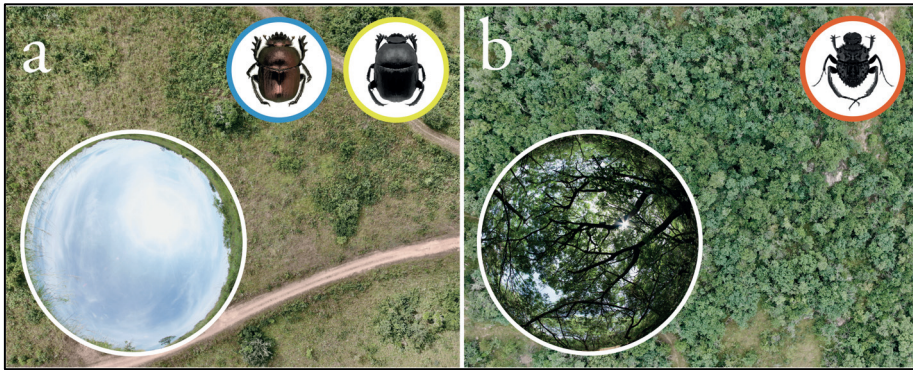


Figure 7. Beetles from three tribes of ball-rollers and the bioregions they inhabit (Paper III). Beetles from three tribes of ball-rollers (*blue circle*: Scarabaeini; *yellow circle*: Gymnopleurini; *orange circle*: Sisyphini) can be found in the same bioregion. *K. nigroaeneus* and *G. unicolor* are predominantly found actively foraging the open region (a) and *S. fasciculatus* predominantly forages within the closed region (b) of this bioregion. A 180° view of the sky as seen from the ground perspective of the beetle is included at the bottom of each panel.

The influence of visual environment on the weight relationship of cues

Since the weight relationship of directional cues in the heading direction network of an animal could depend, in part, on the availability of the cues and the ability of the navigator to detect them, the visual environment is very likely to affect the navigational strategy. For example, desert ant species present in largely landmark-free salt pans, have a higher propensity to rely more on their path integrator over terrestrial cues, while species inhabiting cluttered, landmark-rich environments rely more heavily on landmarks for route guidance (Bühlmann et al., 2011; Cheng et al., 2012). This suggests that the visual ecology of the animal can influence how directional information is weighted in the heading direction network of the animal.

Our knowledge regarding the role of the sun in diurnal dung beetles had, prior to my work in **Paper II**, been limited to species primarily orienting under open, blue skies. However, as ball-rolling beetles are found on all continents except Antarctica, in habitats spanning the deserts of South Africa, to the rainforests of Brazil, I sought out to study the straight-line orientation strategy of beetles in a different visual environment, exploring how the weighting of directional information of the sun in the compass of the dung beetle is influenced by the habitat in which it is active.

The role of the sun in the woodland-living beetle

Contrary to its distant cousins, who are primarily found in dryer, sandy savanna regions (Cambefort and Walter, 1991c), the tiny woodland-living beetle, *Sisyphus fasciculatus*, is often found in more humid and vegetation-rich areas (Daniel et al., 2020; Mucina and Rutherford, 2006; Paschalidis, 1974; Scholtz and Ranwashe, 2020) (**Paper III**) (*Figure 1* and 7). In these types of habitats, the sun is frequently obstructed from view, while wide-field cues (such as the pattern of polarised skylight) remain visible through the overhead canopy (Endler, 1993; Hegedüs et al., 2007; Shashar et al., 1988). Is it possible that beetles active in this type of habitat, attribute the greatest relative weight to directional information provided by this wide-field sky cue, rather than by the sun itself?

To test this hypothesis, I allowed individuals of the beetle *Sisyphus fasciculatus* to steer their dung balls in the presence of a mirrored sun under a clear sky, while simultaneously blocking the real sun from view. In accordance with my hypothesis, the tiny beetles would keep rolling in the same direction as before, completely ignoring the apparent positional change of the sun (*Figure 8a, graph 1*), indicating that the sun compass does not dominate the heading direction network of *S. fasciculatus*.

Considering that nearly all insects, such as bees (Evangelista et al., 2014; Frisch and Lindauer, 1956), ants (Leibhardt et al., 2012; Reid et al., 2011), crickets (Herzmann and Labhart, 1989), locusts (Mappes and Homberg, 2004) and flies (Hardcastle et al., 2020; Warren et al., 2019), derive compass information from the skylight polarisation pattern, a natural second step in my study was to test how the beetles respond to the rotation of the celestial polarisation pattern. Placed underneath a UV transparent polariser, I allowed individuals of *S. fasciculatus* to exit underneath the filter in full view of a clear sky. Prior to the second roll, the polariser was rotated by 90°, in effect turning the artificial band of skylight polarisation. Under this condition, these tiny rollers would change their bearings by approximately 85°, demonstrating that, under the bright, clear sky, with the sun fully available, the heading direction network of these beetles attributed greatest relative weight for guidance to the directional information provided by the pattern of polarised skylight (*Figure 8b, graph 1*). It is however important to note that, under the appropriate circumstances, the woodland-living beetle can indeed obtain directional information from a point-light source. If presented with a single green light spot (a credible replacement for the sun to a beetle (el Jundi et al., 2015a*)), *S. fasciculatus* responds in accordance with a 180° azimuthal displacement of this light (*Figure 8c, graph 1*), by a 185° change in bearing. In summary, my results clearly show that the heading direction network of *S. fasciculatus* attributes greatest relative weight to the directional information provided by the wide-field celestial polarisation pattern, contrary to what has been shown previously in other diurnal beetles.

The findings of **Paper II** ascribe to a weighting strategy similar to that found in the ant; as homing ants, that find their way back home using their path integrator and the guidance of landmarks, are further displaced from their nest, the relative weight of these two reference cues shifts in favour of the former, affording greatest relative weight to the most reliable source of directional information in the habitat (Wystrach et al., 2015). In the case of *S. fasciculatus*, this is naturally the polarised pattern of skylight.

My findings of this little wood-land living dung beetle prompted a deeper investigation into the possible adaptive values of assigning the greater relative weight to directional information provided by a particular celestial cue. In addition to the marked differences in their habitat preference (*Figure 7*), there are distinct inter-tribal discrepancies in body size, eye shape and rolling strategy in the beetle species of these two tribes (Tocco et al., 2019; Tocco et al., 2021) (for an in-depth discussion regarding these properties, see *The influence of noise in a biological compass system* and *The Compound Eyes*) (*Figure 14* and *16*). Owing to, not only the ecological differences, but also the morphological inter-tribal differences, the question arose: Is the relative weight influenced exclusively by the visual ecology of the species?

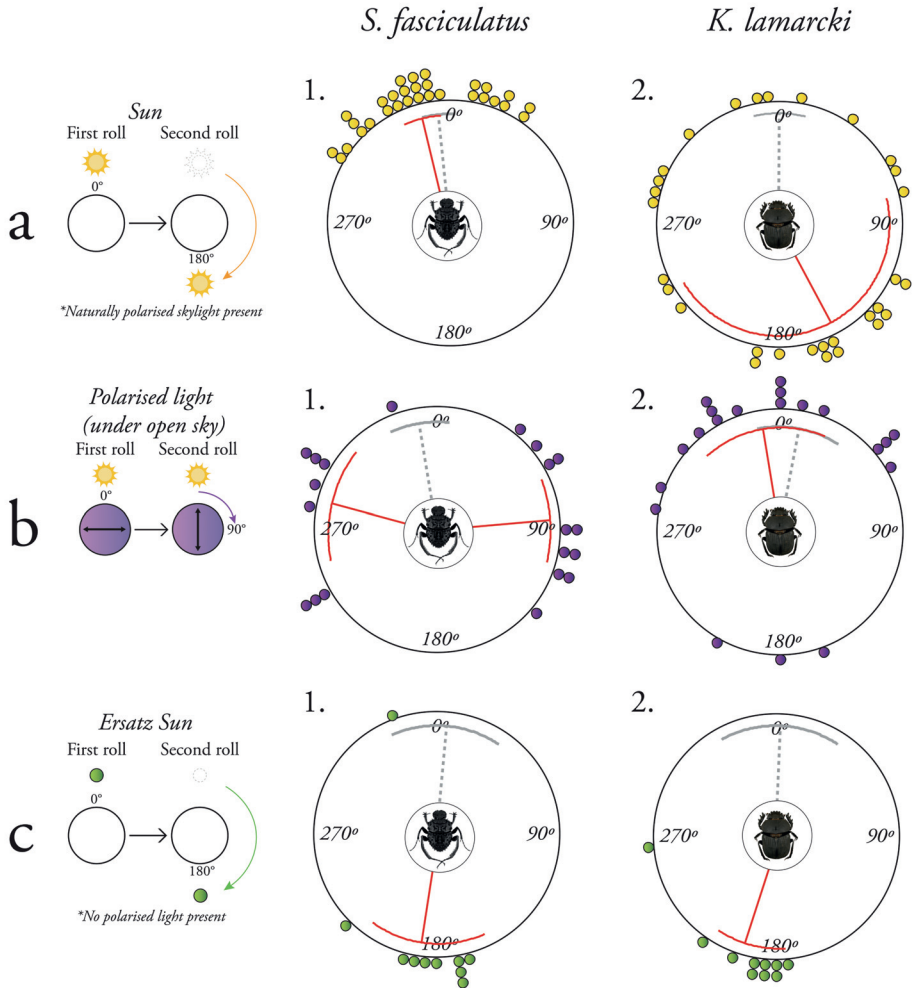


Figure 8. Response to directional changes of compass cues (Paper II). *Sisyphus fasciculatus* and *Kheper lamarcki*, were allowed to roll their dung balls to the perimeter of a circular arena under an open sky (a, b) or in the presence of an artificial sun in an indoor lab (c). Once the beetle had reached the periphery of the arena, it was removed from its dung ball and placed back in the centre alongside its ball. At this time, the position of the tested cue (a: sun, b: polarised light or c: green light spot) was changed. The apparent position of the real sun (a, orange arrow) or the green light spot (c, green arrow) was switched by 180° and the apparent e-vector direction was turned by 90° using a polarisation filter (b, purple arrow). The absolute angular difference between the first and the second exit angle represent the response to the treatment (test) (a, yellow circles; b, purple circles; c, green circles). Beetles were then allowed to roll a third time, with the manipulated cue moved back to its initial position. The mean angular difference between the first and second exit (test), and the first and third exit (control), is represented by a red solid vector and a grey dotted vector, respectively, in each graph. Error bars represents one circular standard deviation.

Directional information from the sun is integrated differently in beetles from different tribes

To answer my questions concerning the possible relationship between the visual ecology of the species and its heading direction network, I next chose to study the straight-line orientation strategy of three species from three different tribes of dung beetles: *Kheper nigroaeneus* [Scarabaeini], *Sisyphus fasciculatus* [Sisyphini] and *Garreta unicolor* [Gymnopleurini], all present in the same region, but actively foraging in vastly different habitats (Figure 7).

To understand the relative weight of directional information from the sun and the celestial polarisation pattern in these three species, I allowed individuals from each species to roll in the presence of a sun, as well as underneath a UV transparent polariser in the presence of the sun. When changing the apparent position of the sun by 180°, only *K. nigroaeneus*, showed a marked response to this change through a changing in heading of 200° (Figure 9b, graph 1). This was not unexpected; although *K. nigroaeneus* is primarily found in a different region to its very close relative, *K. lamarcki* (known to rely primarily on the sun), both species carry large dorsal eyes (the size of the eyes might play a role in weighting strategy: see *The Compound Eyes*) and forage under open, clear skies. Only when the sun was obstructed from the beetle's view, would *K. nigroaeneus* respond to the 90° turn of the polariser with a change in bearing approaching 70° (compare Figure 9c, graph 1 and Figure 9d, graph 1). This is similar to previous results in *K. lamarcki* (el Jundi et al., 2014a); when the predominant cue (the sun) is hidden from view, the beetles respond to the 90° e-vector turn of the polarised skylight. Following this logic, *S. fasciculatus* (which attributes the greatest weight to the skylight polarisation (Paper II)), should not respond to a mirrored sun under a clear sky, but turn with the polariser under the same clear sky. This was exactly what was observed (Figure 9b-c, graph 3).

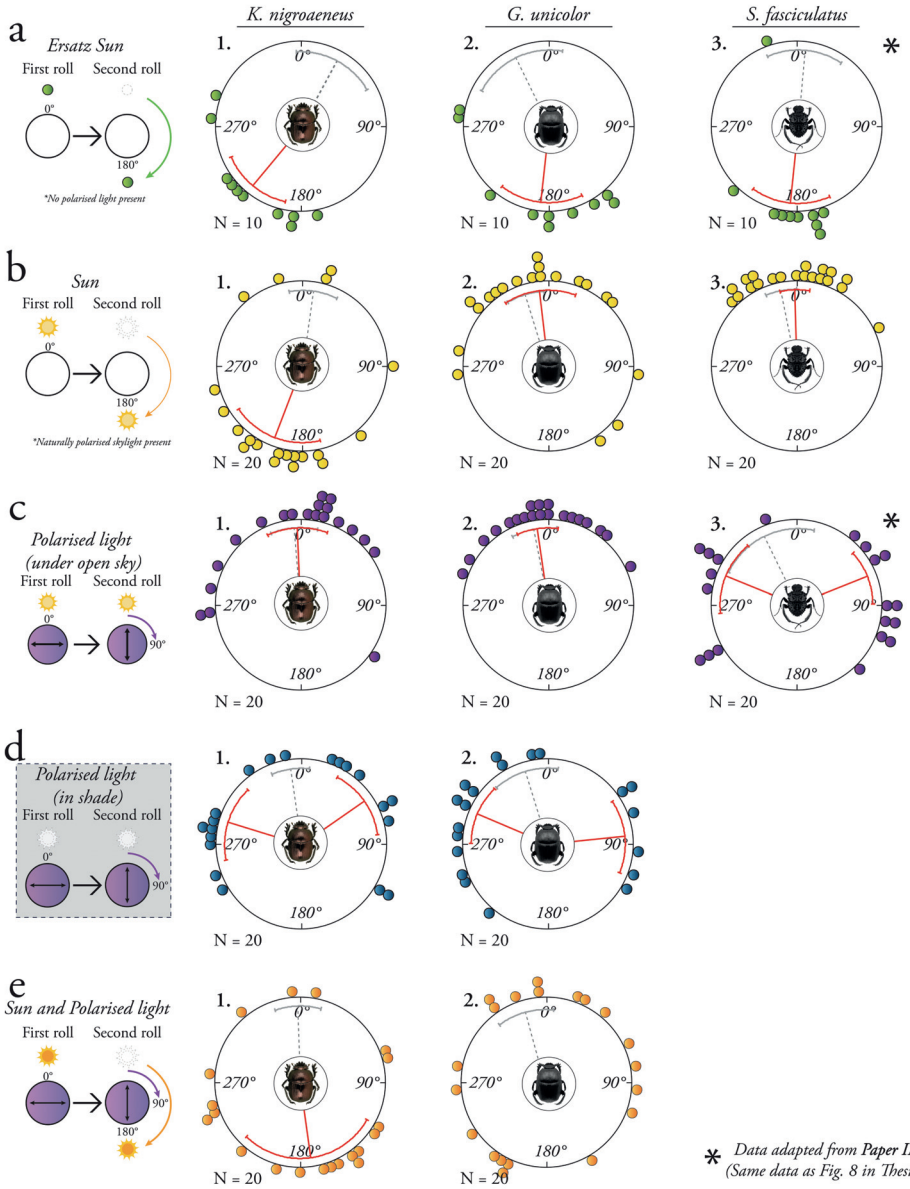
What is interesting is that *Garreta unicolor* (and *G. nitens*, data not shown) did not respond to either of these manipulations (Figure 9b-c, graph 2). Only when the position of both compass cues was manipulated, by mirroring the sun by 180° while simultaneously turning the polariser by 90°, did these beetles change their bearings (Figure 9e, graph 2). However, the response appeared to be randomly distributed. It is important to note that the beetles maintained a straight trajectory when rolling, and as soon as the real sun was revealed and the polariser was turned back to its original position, would the beetles resume their initial direction of travel. In addition, *K. nigroaeneus* was also tested under this multi-conflict paradigm, and showed a clear response to the mirrored sun (Figure 9e, graph 1). Most likely, a similar outcome would have been seen for *S. fasciculatus* but, no matter how hard we tried, we could not manage to get these small beetles to behave under this paradigm. Another important note is that *G. unicolor*, just like *K. nigroaeneus* and *S. fasciculatus*, is able to orient to a

green light spot (*Figure 9a*), indicating that all three beetles can orient to directional information provided by a point-source light.

G. unicolor is found actively foraging under open skies, much like *K. nigroaeneus*, but contrary to its larger distant cousin, it has a narrow, oval-shaped dorsal eye region, much like that found in *S. fasciculatus* (*Figure 14* and *16*). A narrow dorsal eye might encompass a smaller visual field (Cronin et al., 2014; Land, 1997; Land and Nilsson, 2012; Rutowski et al., 2009; Taylor et al., 2019), therefore it could be more beneficial for *S. fasciculatus* and *G. unicolor* to rely on directional information from a sky-wide cue, rather than using the position of a single light source. Thus, these inter-tribal differences might be an additional influence on how species within each tribe weight the sources of directional information they can reliably use higher.

The differing strategy seen in *G. unicolor* can perhaps be explained by the fact that each individual follow their own weighting strategy, wherein some individuals afford greatest relative weight to the sun, turning towards 180° in response to the mirror, some turn with the 90° turn of the polariser, and some remain on the same path, following the remaining sky cues, such as the intensity and colour gradient (see *Spectral and intensity gradient*). However, if this would have been the case, we would have seen a similar spread in bearings in the other conditions as well, which we did not (*Figure 9a-d, graph 2*). Another, more plausible, explanation could be that directional information from the sun and polarised light are near equal in weight, therefore any small individual 'preference' of one cue over the other will be seen as large differences in angular change. It would be of great interest to see how these beetles would respond if both the azimuthal position of the sun and the band of skylight polarisation were rotated by the same angular distance. Would a 90° turn of both cues elicit a 90° turn of the population? Or will the response of the population also here appear random? Unfortunately, due to experimental constraints, this was not possible to test in this study. In **Paper IV** this problem is solved (for more, see *Examining the weight relationship of the sun and polarised skylight by proxy*). For the moment however, the random spread of changes in bearings observed for *G. unicolor* cannot be fully explained.

Change in Bearing



* Data adapted from Paper II (Same data as Fig. 8 in Thesis)

Figure 9. See next page for legend.

Figure 9. Response to directional changes of compass cues (Paper III). Three diurnal dung beetle species (from left to right: *Kheper nigroaeneus*, *Garreta unicolor* and *Sisyphus fasciculatus*) were allowed to roll their balls of dung out of a circular arena in a darkened room (a) or outdoors under the open sky (b), or with a polarisation filter placed above the arena with the sun visible (c, e) or with a polarisation filter placed above the arena with the sun shielded from view (d). Once the beetle had reached the periphery of the arena, it was removed from its dung ball and placed back in the centre alongside its ball. At this time, the apparent position of the ersatz sun (a, green arrow) or the real sun (b, orange arrow) was switched by 180°, the apparent e-vector direction was turned by 90° using a polarisation filter (c-d, purple arrow) or the position of the sun was changed by 180° while simultaneously turning the apparent e-vector direction by 90° (e, orange arrow: sun, purple arrow: polarisation filter). The beetle was then allowed to exit the arena a second time. The absolute angular difference between the first and the second exit angle represent the response to the treatment (*test*). The beetles were then allowed to roll a third time, with the manipulated cue(s) moved back to its/their initial position. The mean angular difference between the first and second exit (*test*), and the first and third exit (*control*), is represented by a red solid vector and a grey dotted vector, respectively, in each graph. Error bars represents one circular standard deviation. The data presented for *S. fasciculatus* in a and c (graph 3, respectively) is adapted from Paper II.

Box 4. The celestial pattern of polarised light

Linear polarised light

The light emitting from the sun is composed of light waves with electric field vectors, *e-vectors*, oscillating in all possible orientations perpendicular to their direction of travel (*Figure 10*). As sunlight reaches the atmosphere, it collides with air molecules and scatters. Following this, the *e-vector* component of the light wave oscillates to a higher degree in one plane over others, becoming partially polarised. The extent to which this component oscillates in one certain direction over others determines the light's *degree of linear polarisation* (Strutt, 1871; Suhai and Horváth, 2004). A light wave that contains an *e-vector* in only one plane is considered fully linearly polarised (100% polarisation). The highest degree of linear polarisation in the sky is present directly perpendicular to the sun and decreases sinusoidally towards the sun and anti-sun (Strutt 1871) (*Figure 10a*).

Although largely invisible to us, many insects, such as dung beetles (**Paper II**; Dacke et al., 2003; Foster et al., 2019*; Jundi et al., 2015a*), ants (Legge et al., 2014; Müller and Wehner, 2007; Wehner, 2003), bees (Evangelista et al., 2014), locusts (Mappes and Homberg, 2004), crickets (Henze and Labhart, 2007), flies (Warren et al., 2019; Weir and Dickinson, 2012) and butterflies (Froy et al., 2003; Heinze and Reppert, 2011; Reppert et al., 2004) perceive this celestial pattern of polarised light through their *dorsal rim area* (a specialized region in the compound eyes of insects (see *The Compound Eyes*), and use it as a directional cue during navigation and orientation.

(continues on next page)

Circularly polarised light

Under certain circumstances, the e-vector component of two light waves can be out of phase. In this case, the resultant e-vector changes its direction over time, resulting in a spiral of the e-vector direction (*Figure 10b*). If the light waves are out of phase by 90° , the light becomes *circularly polarised* (Cronin et al., 2014; Land and Nilsson, 2012). While circularly polarised light does exist in nature, its production and detection are limited to only a few groups of animals, including some scarab beetles and marine stomatopods (Brady and Cummings, 2010; Brink et al., 2007; Chiou et al., 2008). However, as most behaviours known to be controlled by polarised light, are done so by linearly polarised light, all references to polarisation and polarised skylight throughout this thesis refers to its linear form.

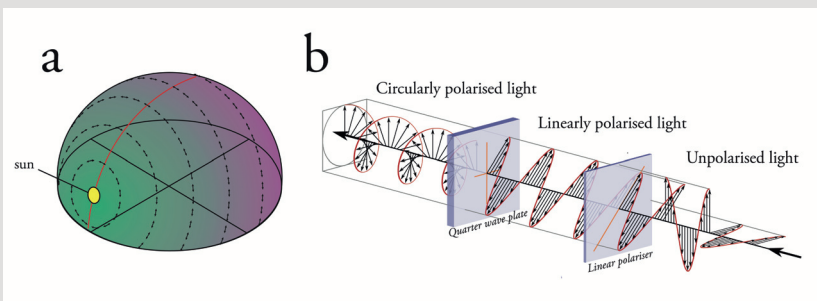


Figure 10. Polarised light and the alignment of e-vectors in relation to the sun.

In the sky, the electric field vectors (*e-vectors*) (*black small arrows*) of the polarised skylight are arranged in concentric circles around the sun in a symmetrical fashion along the solar meridian (*red line*) (a). The maximum degree of polarisation can be found 90° from the sun. In addition, as the solar hemisphere contains a relatively higher amount of long-wavelength light (green), and the antisolar hemisphere contains relatively more short-wavelength light (UV, depicted in the image as purple), a spectral gradient forms across the sky. A schematic drawing of an *unpolarised*, *linearly polarised*, and *circularly polarised* light beam (*orange wave*) is shown in b. The direction of the light beam is depicted by the large black arrow. The e-vector direction along the light beam is depicted by small black arrows. Image in a is modified from *el Jundi et al. 2016*; image in b is modified from <https://commons.wikimedia.org/w/index.php?curid=9862692>.

Polarised Light

The role of polarised light in the heading direction network of the ball-rolling dung beetle

In **Paper III**, when the apparent position of the sun was displaced by 180°, or the apparent e-vector direction of the celestial polarised light was turned by 90°, *Garreta unicolor* did not respond to either of these manipulations. Only when both cues were manipulated simultaneously would *G. unicolor* change its bearing (*Figure 8b-c and e, graph 2*). Based on this curious behaviour of *G. unicolor*, I wanted to further explore the interplay of these two cues in the heading direction network of the dung beetle. More specifically, I aimed to examine how straight-line orientation is affected when the three properties of a polarised light cue (intensity, degree and direction of polarisation) are manipulated and presented with a single, unpolarized light cue (simulating the sun) to the ball-rolling beetle. Could the observed result of *G. unicolor* be a consequence of an equal weighting of directional information of this pair of celestial cues in the heading direction network of the beetle?

Although the obvious choice of species to investigate this interplay would have been *G. unicolor*, geographical constraints, abundance, and overall fussiness of the beetle, makes it a poor candidate for this investigation. Instead, because of its robust nature, calm temperament, and high availability, *Kheper lamarcki* was selected to address this question. In addition, and perhaps more importantly, close to twenty years in the field studying the straight-line orientation of this species have provided a plethora of knowledge regarding its orientation system, on a behavioural as well as neuronal level, making it a superb candidate for my last study of this thesis.

Examining the weight relationship of the sun and polarised skylight by proxy

Exploring the interplay of the sun and the polarised skylight under natural conditions presents some obvious challenges. First, as discussed previously for **Paper III**, changing the azimuthal position of both cues equally, i.e., turning the apparent position of the sun by 90° to match the maximum possible angular change of the e-vector, presents its own set of challenges. Second, manipulating the reliability of each cue without compromising the properties of the remaining celestial cues is a difficult (if not impossible) task. Third, there are significant restrictions regarding the properties that can be manipulated. Across a bright savanna sky, the sun and the polarised skylight has virtually reached their upper limit concerning light intensity and degree of polarisation (Foster et al., 2019*; Johnsen et al., 2006; Lythgoe, 1979; Warrant et al., 2020). Due

to these constraints, in **Paper IV**, I introduced *K. lamarcki* to artificial compass cues as a proxy for the sun and the polarised skylight pattern (*Figure 11*). By altering the qualities of the polarised light cue, this allowed me to examine how the weight relationship of these two sources is influenced and altered by their reliability in the heading direction network of the beetle.

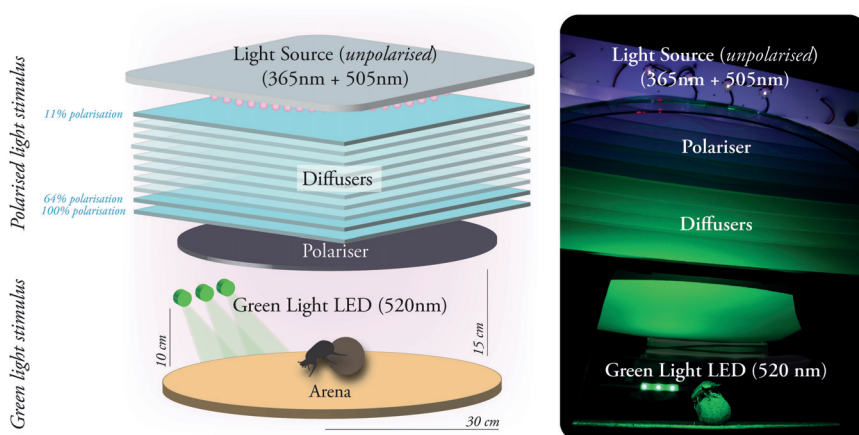


Figure 11. Description of the experimental setup of Paper IV. The experimental setup combined an overhead *Polarised light stimulus* and a laterally presented *Green light stimulus*. The overhead light stimulus was suspended 15 cm above a circular arena of 30 cm radius and consisted of an unpolarised light fixture ('Light Source') of 80 UV light-emitting diodes (365 nm) and 21 cyan light-emitting diodes (510 nm) centred on a square shaped aluminium plate, along with ten sheets of 'Diffusers' (Plexiglas) arranged in a stacked fashion 1 cm apart, and a polarisation filter ('Polariser'). Depending on the placement of the polarisation filter within the stack of diffuser, the degree of polarisation of light emitted from the overhead light source could be determined. For my experimental purposes, the polariser was placed in three different positions within the setup (highlighted in blue in the figure): *i*) before the stack of diffusers (11 % polarisation), *ii*) before the 9th diffuser (64 % polarisation), or *iii*) after the stack of diffusers (100 % polarisation). The green unpolarised light stimulus (520 nm) consisted of 3 horizontally aligned LEDs (9.5 cm x 0.5 cm) presented to the beetle from either of the four sides of the arena (0°, 90°, 180° and 270°), 30 cm from the arena centre, at a height of 10 cm. *Left ima*

ge: schematic depiction of the experimental setup. *Right image*: real image of the experimental setup.

*Evidence for two spectrally distinct photoreceptors in the dorsal rim area of *Kheper lamarcki**

Across the clear, sun-lit sky, the celestial polarisation pattern is highly distinguishable over all wavelengths of light. However, if observed underneath a tree canopy or experienced through a layer of clouds, the maximal degree of polarisation is in the UV range (Barta and Horváth, 2004; Hegedüs et al., 2007; Seliger et al., 1994; Wang et al., 2014). Perhaps, this is why most insects, including honeybees, ants, maybeetles, butterflies and flies (Duelli and Wehner, 1973; Frantsevich et al., 1977; Hardie et al.,

1979; Labhart, 1980; Stalleicken et al., 2006) analyse this pattern through a set of UV sensitive photoreceptors. Interestingly, for the diurnal dung beetle *K. lamarcki*, the findings of my co-authors Ayse Yilmaz (Lund University, Sweden) and Gregor Belušič (University of Ljubljana, Slovenia) in **Paper IV** show evidence for polarisation sensitivity in the UV *as well as* the green part of the spectrum (*Figure 12*). This relatively rare finding of two spectrally distinct, polarisation-sensitive types of photoreceptors, has also been observed in the diurnal homing dung beetle, *Pachysoma striatum* (Dacke et al., 2002). This type of spectral sensitivity across a broader spectrum of light can for example favour the absolute polarisation sensitivity of the eyes (Belušič et al., 2017; Eggers and Gewecke, 1993; Labhart et al., 1992). For experimental purposes, the artificial overhead polarised light source presented to the beetles in **Paper IV** was designed to match the maxima of the two spectral sensitivity peaks of the photoreceptors in the dorsal region of the dorsal eye of *K. lamarcki* (*Figure 11* and *12*).

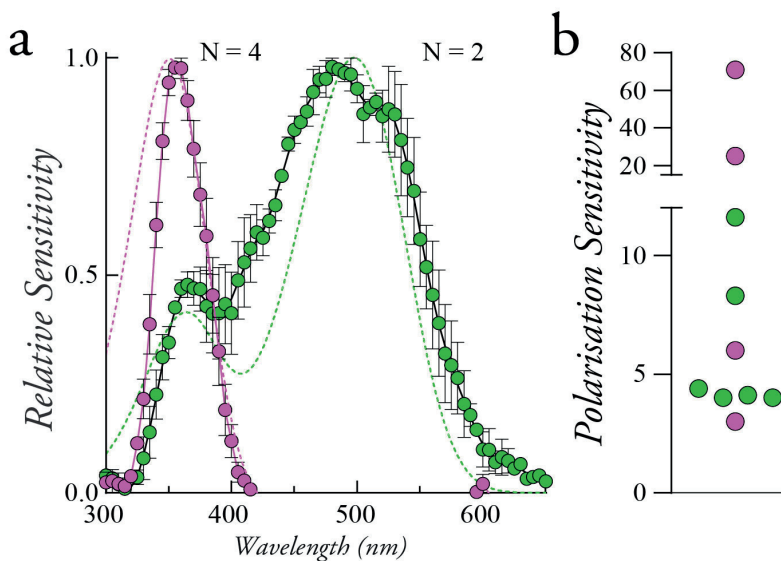


Figure 12. Spectral sensitivity of photoreceptors in the dorsal region of the dorsal eye of *Kheper lamarcki* (**Paper IV**). Intracellular recordings in the dorsal region of the dorsal eye of *K. lamarcki* revealed two types of spectrally distinct, but highly polarisation sensitive photoreceptors (a). The spectral sensitivities of the ultraviolet sensitive photoreceptor ($\lambda_{\max} \approx 350$ nm) (*pink graph*) and the green sensitive photoreceptor ($\lambda_{\max} \approx 500$ nm) (*green graph*) are fitted with rhodopsin nomograms (*pink dashed line*: $\lambda_{\max} = 352$ nm; *green dashed line*: $\lambda_{\max} = 501$ nm). The range of measured polarisation sensitivities of the ultraviolet (*pink circles*) and green (*green circles*) photoreceptors can be seen in b. Courtesy of Ayse Yilmaz and Gregor Belušič.

Directional information is weighted according to Bayesian reasoning

As the degree of polarised light is determined by the intensity of the electric field component in proportion to the light beam's overall intensity (Strutt, 1871; Suhai and Horváth, 2004) (*Box 4*), this is also a measure of signal strength: the higher the degree of polarisation, the stronger the signal. It is thus plausible to assume that the reliability of the polarised light source as a directional cue decreases as a function of its degree. When beetles of *K. lamarcki* were allowed to roll under an overhead polarised light source of three differing degrees of polarisation (100%, 64% or 11%) (*Figure 11*) of equal intensity, they showed a clear response to the 90° rotation of the artificial band of polarised light under all three degrees of polarisation (*Figure 13, b-d*) (**Paper IV**). In addition, our data revealed a significant correlation between the degree of polarisation and the probability of a turn (45° or more), demonstrating that turning probability decreases with decreasing degree of polarisation. A similar trend is also observed in crickets, where the polarotactic response diminishes as the animal is presented a stimulus of lower degree of polarisation (Henze and Labhart, 2007; Labhart, 1996).

Interestingly, my results from **Paper IV** revealed that *K. lamarcki* can extract and utilise directional information from a degree of polarisation as low as 11% (*Figure 13d*), a value corresponding to the threshold limit suggested for its nocturnal cousin, *Scarabaeus satyrus* Fabricius (Foster et al., 2019*). However, it is important to note that the light intensity presented to the diurnal beetle in **Paper IV** is four orders of magnitude higher than that presented to *S. satyrus* in Foster et al. 2019*, and nearly three orders of magnitude higher than the intensity of polarised light in the diurnal sky (Johnsen et al., 2006). Although the light intensity under a moon-lit night is significantly dimmer (approximately six orders of magnitude darker (Johnsen et al., 2006; Lythgoe, 1979), the degree of polarisation is comparable to a diurnal sky, with approximately 60% polarisation of the skylight (Foster et al., 2019*; Horváth et al., 2014). If the heading direction network of the beetle only evaluates the degree of the polarised light cue for directional information, *K. lamarcki* should in theory also be able to steer in reference to the night sky polarisation. Coerced to roll during a moon-lit night, with the apparent position of the real moon covered from the beetle's field of view, these diurnal beetles do, however, fail to maintain a straight bearing (Smolka et al., 2016). This indicates that the lowest detectable intensity (but not degree) of polarised light varies between the species.

When introducing and rotating a green light spot in combination with an overhead polarised light source at 11% polarisation, *K. lamarcki* turned in response to the 90° displacement of the green light (*Figure 13g*). It appears that this single bright spot generates a stronger and more reliable directional signal relative to the artificial band of polarised light. This weighting relationship is directly comparable to that observed outdoors; when the apparent position of the sun is changed by 180° with the aid of a mirror, while simultaneously blocking the real sun from view under a natural sky, *K.*

lamarcki will turn in response to the mirrored sun (see *Compass cues used by the ball-rolling dung beetle*). When instead presented with a fully (100%) polarised light source in the lab, in addition to the same laterally presented green light source as above, the beetles no longer turned in response to a 90° azimuthal change of the green light (*Figure 13e*). Instead, they now steer straight according to the polarised light stimulus. This demonstrates that *K. lamarcki* alters its weighting of directional cues in a stimulus-dependent manner to steer straight. That is, as the relative strength or reliability of the directional cues change, the relative weighting of these cues in the heading direction network of the beetle changes accordingly.

The weighting strategy of the beetle appears to be performed according to Bayesian reasoning (Körding, 2007; Körding and Wolpert, 2006). According to this theory, directional information conveying the highest certainty at the given moment is afforded the greatest weight in the navigational system of the animal. This influence of reliability on weighting strategy is also apparent in homing ants, that will rely more on path integration over terrestrial cues at visually unfamiliar locations (Wystrach et al., 2015), and vice versa when located in more familiar terrain (Legge et al., 2014). Similarly, sandhoppers, that predominantly rely on directional information from the sun (Ugolini et al., 2002; Ugolini et al., 2015; Ugolini et al., 2016), will utilise radiance and spectral gradient cues to direct their movements when this celestial body is out of sight (Ciofini et al., 2021), and *Myrmica* ants, that rely on visual guidance for navigation, resort to olfactory cues as the light intensity decreases (Cammaerts, 2012).

Along the same line of reasoning, following a ‘Bayesian weight strategy’, this would also imply that when two directional cues of equal weight are set in conflict with each other, an intermediate heading direction to that of both sources of directional information is chosen. Although only hypothesized for *G. unicolor* in **Paper III**, this outcome has been repeatedly observed in ants: when the apparent e-vector direction of the celestial pattern of polarised light is set in conflict with the artificial panorama (Freas et al., 2017; Reid et al., 2011) or the artificial panorama is set in conflict with celestial cues (Legge et al., 2014; Wystrach et al., 2015), these insects choose an intermediate bearing to the dictates of both sources of directional information. When ball-rolling *K. lamarcki* was presented with a 64% polarised overhead light in combination with a green light spot, the beetles changed their bearings by about 45° in response to a 90° rotation of the light (*Figure 13f*). This could be because the two sources of input signals under this paradigm are providing directional information of similar reliability. Interestingly, as soon as the intensity of the overhead polarised light was lowered, the beetles again turned with the ersatz sun (*Figure 13h*), demonstrating that weakening the relative input of directional information from the polarisation cue again effectively shifted the relative weighting towards the ersatz sun.

From my results in **Paper IV**, I can safely conclude that *K. lamarcki* integrates multiple sources of directional information in a Bayesian manner (Cheng et al., 2007; Körding, 2007; Körding and Wolpert, 2006), strongly suggesting that directional

information conveying the highest certainty at any given moment is afforded the greatest weight in the navigational network of the animal.

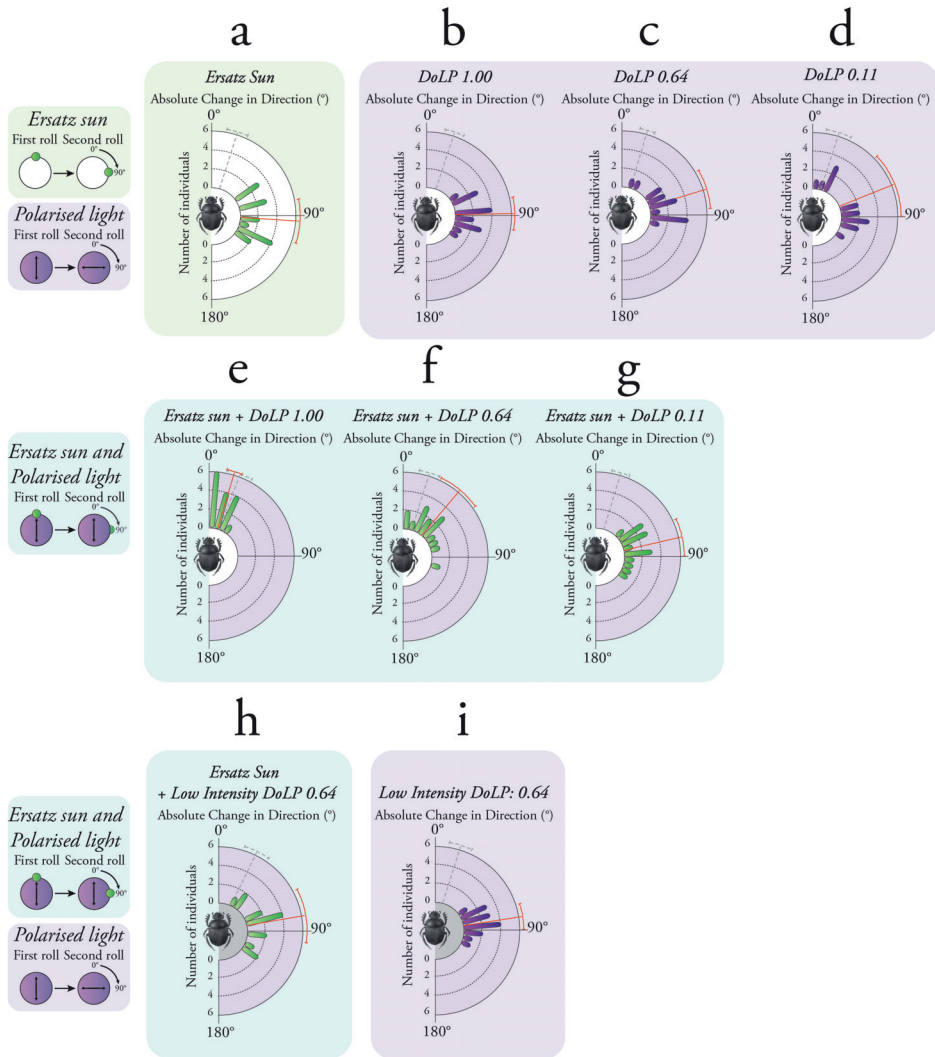


Figure 13. See next page for legend.

Figure 13. Response to directional changes of compass cues (Paper IV). *Kheper lamarcki* was allowed to roll its dung ball from the centre of a 30 cm radius arena presented with a lateral green light source (ersatz sun) in the presence of an overhead unpolarised light source (a), a single overhead polarised light source (b: 100% polarisation; c: 64% polarisation; d: 11% polarisation), a laterally presented green light source in combination with an overhead polarised light (e: ersatz sun in the presence of 100% polarisation; f: ersatz sun in the presence of 64% polarisation; g: ersatz sun in the presence of 11% polarisation; h: ersatz sun in the presence of 64% polarisation of lower light intensity), or in the presence of a single overhead polarised light source of 64% polarisation of lower light intensity (i). Once the beetle had reached the periphery of the arena, it was removed from its dung ball and placed back in the centre alongside its ball. This procedure was repeated five times. After the fifth exit from the arena, the apparent position of the ersatz sun (a, e-h) or the e-vector direction of the artificial band of the overhead polarised light source (b-d, i) was turned by 90° (*test*), or remained in position (*control*) and the beetle was allowed to exit the arena again for five consecutive rolls. The absolute angular change between the mean direction of the five rolls prior to the treatment and the mean direction of the five rolls preceding the treatment in the test condition is depicted as coloured bars in all graphs. When a single stimulus was presented in isolation (a-d), *K. lamarcki* changed bearing direction in accordance with the 90° angular change of the stimulus presented (*red vector*, all graphs). When rolling in the presence of an ersatz sun under 100% polarised light, *K. lamarcki* did not respond to the positional change of the ersatz sun (e). Contrarily, when rolling in the presence of an ersatz sun under 11% polarised light, *K. lamarcki* changed its bearing in accordance with the 90° angular turn of the ersatz sun (g). If presented with an ersatz sun in the presence of 64% polarised light, this beetle showed an intermediate response to the azimuthal change of the stimulus (f). However, when the intensity of the 64% polarised light decreased by tenfold, the beetle would now turn in response to the 90° turn of the ersatz sun (i). The absolute angular difference between the mean direction of the five rolls prior to the treatment and the five rolls preceding the treatment during the control condition is represented by a grey dotted vector in each graph. Error bars represent one circular standard deviation.

Spectral and intensity gradient

Spectral skylight and the intensity gradient of the sky as directional information

The atmospheric scattering of sunlight also generates a spectral (and intensity) gradient across the sky (*Figure 10a*). With increasing angular distance from the sun, the dominant wavelength of light decreases (Strutt, 1871), creating a chromatic contrast between long and short wavelength of light along the solar meridian. Consequently, the relative intensity of green light is stronger in the direction of the sun, and the relative intensity of ultraviolet (UV) light is stronger towards the antisolar direction (*Figure 10a*) (Coemans et al., 1994). This gradient can be exploited as a directional reference; honeybees are able to distinguish between the sun and the antisolar direction in the sky by associating long wavelengths to the solar half, while short wavelengths are associated to the antisolar half (Edrich et al., 1979; Rossel and Wehner, 1984) and sandhoppers are able to orient to the correct seaward direction of their home beach using the spectral gradient (Ciofini et al., 2021). The ability to derive compass information from spectral cues has also been demonstrated in the ball-rolling dung beetle *Kheper lamarcki* (el Jundi et al. 2015a*; el Jundi et al., 2016*). When presented with two green light spots positioned 180° apart, the beetles could not adhere to the same original direction over two consecutive rolls. However, if presented with one green light spot and one UV light spot, positioned 180° apart, the beetles had no difficulty in maintaining their bearing over two rolls. Their ability to adhere to the same direction persisted even when one of the light spots (UV or green) were turned off prior to the second roll. This clearly demonstrates that the beetles consider the position of both spectral cues in relation to one another, and shows that they can, much like honeybees and sandhoppers, use the celestial chromatic contrast for orientation. Dung beetles are also able to orient by the celestial light intensity gradient. If allowed to roll under an intensity gradient filter (a filter that becomes progressively darker from one side to the other) with the sun and the polarised skylight blocked from view (with the aid of a shading board and a depolariser, respectively), the dung beetle will turn in response to the 180° turn of the intensity gradient filter, demonstrating that *K. lamarcki* is able to use the light intensity gradient as an orientation cue (el Jundi et al., 2014a).

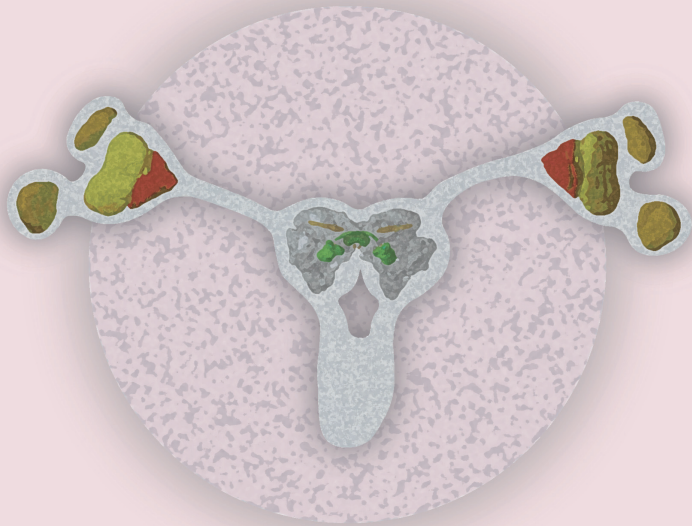
In principle, spectral and/or intensity gradient information only provides the navigator with information about whether it is moving towards or away from the solar hemisphere. Therefore, it is a great supplement to a more robust compass (el Jundi et al., 2014a), rather than a primary source of directional information. It might be for this reason there are, as far as I know, no insects that rely primarily on this type of directional information. Instead, most insects will exploit the spectral and light intensity gradient

of skylight in addition to other cues, such as the sun and polarised skylight (reviewed by el Jundi et al. 2014b).

Wind

Dung beetles can utilise directional information from wind for straight-line orientation

Although repeatedly demonstrated that ball-rolling dung beetles utilise directional information from celestial cues to perform straight-line orientation (see previous sections), these peculiar insects are also able to extract directional information from the wind. If presented with a wind cue (or air accelerated by a fan) in the presence of the sun (or a single green light spot), dung beetles will increasingly rely on directional information from the wind as the sun is approaching zenith (Dacke et al., 2019; Shaverdian, Dirlik, Mitchell et al., 2021, *in prep*). This indicates that directional cue information can be transferred over several modalities, and much like has been observed in **Paper IV**, the beetles effectively shift the weight relationship towards the more reliable source of directional information, regardless of modality. This is also observed in ants, where, if experimentally deprived of their polarisation information, the weight relationship of the directional information from the sun and wind cue shifts in favour of wind as the solar elevation increases (Müller and Wehner, 2007).



The compass pathway: from visual input to behavioural output

Having provided a fair overview of the behaviour of the ball-rolling dung beetle, and the directional information utilised and integrated by the dung beetle compass as the beetle is rolling, I have yet to describe *how* this information is believed to be integrated. For this, we need to start at the beginning, where the directional information is first detected by the compass: the compound eyes.

The compound eyes

Compass cue information is first processed through the compound eyes of the dung beetle

As the beetle gets ready to depart with its dung ball from the pat, it is believed to register the compass information in the sky (Baird et al. 2010; el Jundi et al. 2016*) through its compound eyes (Byrne and Dacke 2011). Most ball-rolling dung beetles have four eyes (two dorsal, and two ventral) separated by a *canthus* (Figure 14). There are, however, some species of ball-rollers, such as *Sisyphus fasciculatus*, *Garreta unicolor* and *G. nitens* that possess only one pair of eyes. In these beetles, the dorsal and ventral regions of the eyes are only partially separated by the canthus (Paper III; Tocco et al., 2019) (Figure 14). At the time of this thesis, it is not fully understood what role the morphology of the eye plays in the orientation behaviour of the beetle (Paper II and III).

The compound eye comprises several optical units, **ommatidia** (Figure 15), that together form the image of the world perceived by the insect. The number of optical units in an eye varies across different groups of arthropods, ranging from very few, such as the 22 ommatidia in the eye of the water flea *Daphnia magna* (Smith and Macagno

1990), to the approximate thousand carried by the ball-rolling dung beetle (Byrne and Dacke 2011), to over tens of thousands, as in the eye of a dragonfly (Sherk 1978).

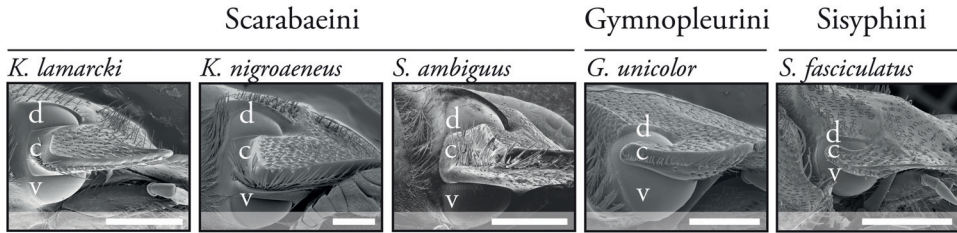


Figure 14. External eye design in three tribes of ball-rolling dung beetles. To facilitate straight-line orientation, diurnal species within the tribe Scarabaeini under most natural conditions attribute the highest weight to directional information provided by the sun. In contrast, *Sisyphus fasciculatus*, from the tribe Sisyphini, relies predominantly on polarised skylight for directional information. For beetles from the tribe Gymnopleurini, neither directional information from the sun nor from the polarisation pattern dominates the output from its compass network (Paper III). The canthus (*c*) completely separates the dorsal (*d*) and ventral (*v*) eyes of Scarabaeini spp., while the only partially separated dorsal part of the eyes of Gymnopleurini spp. and Sisyphini spp. are significantly smaller than their ventral parts. Scale bar is 1mm.

The compound eye functions as a light gathering unit

In general, there are two main types of compound eyes; *apposition compound eyes* and *superposition compound eyes*. In the **apposition eye**, each ommatidium is optically isolated from the other. As follows, each rhabdom receives light through the facet of one single ommatidium, forming an image of only a small part of the visual field of the entire eye. Together, the light gathering units of the eye create a mosaic of the whole visual field. In a **superposition eye**, there is a space between the facet and its rhabdom in the form of a *clear zone* (Figure 15). Here, light from one certain direction can be imaged onto neighbouring ommatidia. Superposition eyes are very common in nocturnal insects, as it supports higher summation of light per rhabdom, making the eye more light-sensitive in contrast to the apposition eye (Land and Nilsson, 2012; Cronin et al. 2014). However, superposition eyes are not limited to nocturnal insects, but can also be found in diurnal species, including ball-rolling dung beetles (Paper III; Byrne and Dacke 2011) (Figure 14 and 16), hawkmoths (Warrant, Bartsch and Günther, 1999) and owl flies (Belušič G, Pirih P, Stavenga DG, 2013).

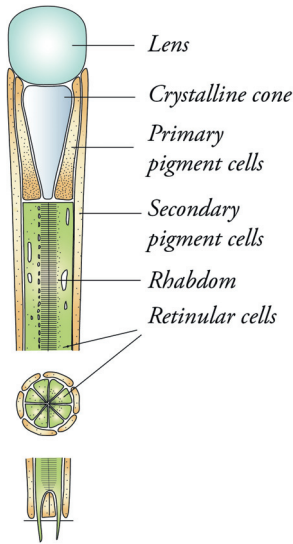


Figure 15. Schematic drawing of an ommatidium. Visual information in the form of light reaches the lens of the ommatidium and travels down to the rhabdom via the crystalline cone. Pigment cells lining the walls of the ommatidium help prevent incoming light from spreading to neighbouring ommatidia. In the rhabdom, the absorption of light initiates an electrical signal that is sent to the brain for further processing. Adapted from Cronin et al. (2014).

The rhabdom detects the light and converts it to a neural signal

As light from the outside world reaches the compound eye of the beetle, the light first passes the lens of the ommatidium, travelling down through the crystalline cone underneath, and finally reaches the **rhabdom**. While the lens and crystalline cone can be thought of as the ‘light gathering’ unit of the ommatidium, the rhabdom functions as the ‘light detecting’ unit. It is in this part of the eye that incoming light is translated to a neural signal. The rhabdom is (usually) formed by seven or eight *photoreceptor cells* (or retinular cells) (*Figure 16*) in which the microvillar component of each of these cells run along its entirety and is oriented inwards, towards the centre of the rhabdom (*Figure 15*). These microvilli all contain light sensitive pigments, *rhodopsin*, composed of an opsin protein bound to a light-absorbing chromophore. Depending on the configuration of opsin and chromophore type, the visual pigment will be more or less sensitive to certain wavelengths of light, affectively determining the wavelength sensitivity of the entire photoreceptor cell.

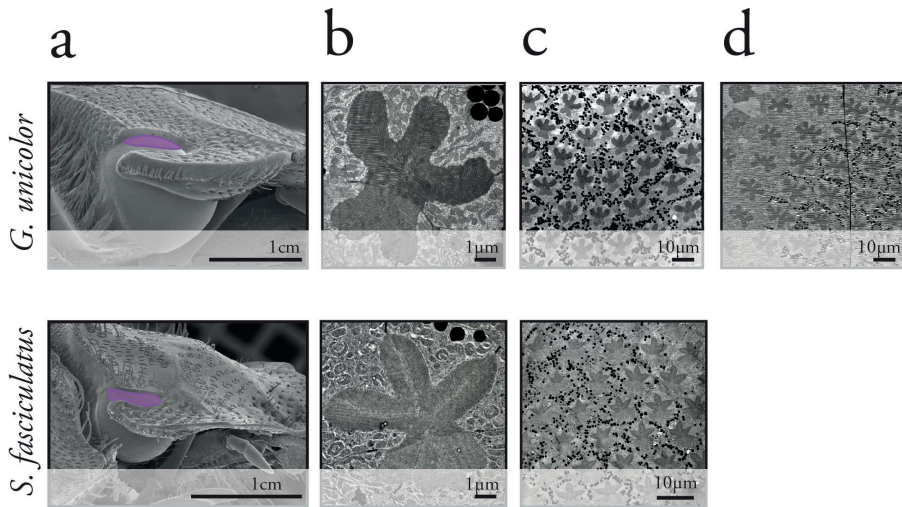


Figure 16. Rhabdoms in the dorsal region of the dorsal eye of *Garreta unicolor* and *Sisyphus fasciculatus*. The rhabdoms of dung beetle eyes are formed by seven receptor cells and differ in shape and microvillar orientation in different eye regions. The rhabdoms of the dorsal rim area (DRA) (b and c) possess orthogonally arranged microvilli, while the rhabdoms in the rest of the dorsal eye (d) are flower shaped with varying microvillar orientations. Based on my preliminary data, the presumed DRA of each species is highlighted in purple in a.

The opsins are the building blocks of wavelength sensitivity

Despite the incredible diversity within the insect realm, the wavelength sensitivity of arthropod photoreceptors is quite conservative. In general, insects possess three types of **opsin groups** of different wavelength sensitivities, with absorbance peaks in the *ultraviolet* (also known as *short*, ranging around 350 nm), *blue* (also known as *medium*, ranging around 440 nm) and *green* (also known as *long*, ranging around 520 nm) wavelength range (Briscoe and Chittka, 2001). Throughout evolution, the possession of these three opsin paralogues have been modified, and in some insects, such as the dung beetle, only two out of the three opsin types, effectively the UV and long wavelength opsins remain (Paper IV; Lord et al., 2016; Sharkey et al., 2017) (Figure 12).

The dorsal rim area detects polarised light

The majority of an insect compound eye consists of rhabdoms with microvilli arranged in all different directions (Figure 15 and 16d). In addition to this, many insects have a region of specialized ommatidia commonly found in the most dorsal part of the eye. In this region, termed the **dorsal rim area** (DRA) (Figure 16b-c), the microvilli of each

photoreceptor within the rhabdom are distributed exclusively in two different directions, orthogonal to each other (*Figure 16b*). This causes each photoreceptor cell to be sensitive to light oscillating in one specific plane (*Figure 10b*, also see *Box 4*), essentially forming a polarisation detector in each ommatidium.

Most insects (Labhart and Meyer, 1999), including dung beetles (Dacke et al., 2002; Dacke et al., 2003), crickets (Brunner and Labhart, 1987), honeybees (Wehner and Strasser, 1985) and ants (Labhart, 1986) possess compound eyes with a DRA. For many of these insects, the photoreceptor cells within this region are UV wavelength sensitive (Labhart, 1986; Nilsson et al., 1987). However, in some insects, such as in crickets (Herzmann and Labhart, 1989) and locusts (Schmeling et al., 2014), the DRA presents blue wavelength sensitive photoreceptors. Although less common (Hegedüs et al., 2006), green wavelength sensitive photoreceptors in the DRA have also been found in the cockchafer (Labhart et al., 1992) and very recently also in the diurnal beetle, *Xheper lamarcki* (**Paper IV**) and the nocturnal beetle, *Scarabaeus satyrus* (Yilmaz, *unpublished*).

The size of the dorsal rim area affects the polarisation sensitivity of the dung beetle

Prior to this thesis, all studies of straight-line orientation in diurnal ball-rolling beetles have shown that diurnal ball-rollers attribute greatest relative weight to the directional information provided by the sun (**Paper I**; **Paper II**; **Paper III**; Byrne et al., 2003; Dacke et al., 2014; Dacke et al., 2019; el Jundi et al., 2015a*). However, if the sun gets out of view, due to cloud cover or vegetation, the beetles continue to steer straight according to the celestial pattern of polarised light (Jundi et al., 2014a) or the colour and intensity gradient spanning across the sky (Jundi et al., 2014a; el Jundi et al., 2015b), with no decrease in performance.

In **Paper II** and **III** I showed that when changing the position of the apparent sun by 180°, while shading the real sun from view, under the natural sky, *Sisyphus fasciculatus* along with *Garreta unicolor*, remained unresponsive to this change. A possible rationale for this could be the simple constraints of the external eye: both species possess much narrower dorsal eyes compared to that of any other diurnal dung beetle studied in this thesis (*Figure 14*). A smaller compound eye could possibly contribute to a smaller visual field (Cronin et al., 2014; Land, 1997; Land and Nilsson, 2012; Rutowski et al., 2009; Taylor et al., 2019). Hence, it might be more beneficial to integrate information from a sky-wide signal, such as the celestial polarisation pattern, rather than determining the position of a single light source. Although yet to be confirmed, the mentioned beetles, specifically *S. fasciculatus* – which clearly weights directional information from polarised skylight over the sun (**Paper II**), could also possess a DRA covering a larger part of the dorsal eye (see *Figure 16*). A narrow dorsal eye limiting the visual field of the beetle, in combination with a large DRA could

support a higher sensitivity of polarised light input, contributing to the behavioural responses documented for *S. fasciculatus* and *G. unicolor*.

It is important to note that the size of the DRA does not necessarily correlate with the sensitivity to polarised skylight in insects (Labhart and Meyer, 1999), however, based on past and previous behavioural observations in the dung beetle, the size of the DRA in the dung beetle is very likely to influence the weighting strategy of the animal's orientation system. This is at least observed for the nocturnal and crepuscular beetles, *Scarabaeus satyrus* and *Scarabaeus zambesianus* Péringuey. These animals rely on directional information from the polarised skylight above that from the moon (Dacke et al., 2004; Dacke et al., 2003; Dacke et al., 2011; Dacke et al., 2013b; Foster et al., 2019*). Interestingly, if instead coerced to roll during the day, the nocturnal *S. satyrus* will weight directional information from the sun over that provided by the celestial polarised light pattern (el Jundi et al., 2015a*). It is now most likely the drastic increase in light intensity (Lythgoe, 1979) that changes the weight relationship of the directional cues in the neural network of the beetle (**Paper IV**, *Figure 13*, and *The brain*). Comparably, if the diurnal analogous, *Kheper lamarcki*, is coerced to roll at night, it still primarily follows the directional information provided by a point-source light, i.e. the moon. It can be so that the eyes of *K. lamarcki* are just not be able to detect the polarised skylight pattern (or any other additional celestial cues) across the night sky. Only a single row of DRA has been found in *Kheper lamarcki* (Dacke, *unpublished*). This limited region of DRA, in combination with a subpar adaptation to dim light vision (Dacke et al., 2011; Smolka et al., 2016), can be contributing factors that constrain the beetle's ability to utilise the nocturnal celestial polarisation pattern as it rolls under the night sky.

The brain

The pathway to the central complex

Unlike locusts, monarchs and Bogong moths, that migrate over tremendous distances (Homberg, 2015; Perez et al., 1997; Warrant et al., 2016), or ants, bees and wasps, that need to return to their nests after a successful foraging trip (Collett, 2012; Mandal et al., 2017; Rossel and Wehner, 1984; Wehner, 1996; Wehner, 2003), the ball-rolling dung beetle ‘simply’ needs to move away from a single point in as straight of a trajectory as possible, for the duration of approximately 6 minutes (Dacke et al., 2019). Nonetheless, different navigational strategies aside, the neural pathway underlying these behaviours are highly conserved across the entire insect realm (Heinze and Pfeiffer, 2018; Homberg et al., 2011; Ito et al., 2014; el Jundi et al., 2018; Pfeiffer and Homberg, 2013; Stone et al., 2017; Zeller et al., 2015), strongly suggesting that all travelling insects rely on the same neural basis to achieve their guided movements.

Prior to rolling, the beetle climbs on top of its ball and begins to rotate around its vertical axis in a motion reminiscent of the learning walk pirouettes of ants (see Grob et al., 2019; Lent et al., 2010; Zeil and Fleischmann, 2019). During this pirouette, or dance, the beetle is believed to take a snapshot of the sky, that it then attempts to continuously match while rolling (Baird et al., 2012; el Jundi et al., 2016*). As the beetle dances, light reaches the photoreceptors and swiftly converts into neural signals, that in turn is transferred to the first processing centre for visual input; **the optic lobe** (Immonen et al. 2017). Here, the neural signal is received by the first two neuropils of the optic lobe; *the lamina*, proximally followed by *the medulla* (*Figure 17*), where polarised light information from the dorsal rim area, along with unpolarised light information from the rest of the retina are integrated and transferred by transmedulla neurons to the central brain, specifically **the anterior optic tubercle** (el Jundi, Pfeiffer and Homberg, 2011; Blum and Labhart, 2000; Kinoshita, Pfeiffer and Homberg, 2007; Homberg and Paech, 2002). Interestingly, in the dung beetle, the transmedulla neurons only project from the dorsal medulla, indicating that celestial information is only received from the dorsal eyes of the beetle (most dung beetles have four eyes, see *The Compound Eyes* and *Figure 14*) (Immonen et al. 2017). This neuroanatomical finding is very much reflected in the behavioural output of the beetle: when the diurnal dung beetle, *Kheper lamarcki*, is allowed to roll under an open sky, with an opaque black cap shielding its dorsal eyes from viewing the sky, it is unsuccessful in maintaining a straight trajectory (Dacke et al., 2013b), indicating the need of dorsal light input for orientation. After the anterior optic tubercle, the neural signal from the eye is carried to **the ipsilateral bulb of the lateral complex** (el Jundi et al., 2018). This is considered the ‘last destination’ prior to the brain region believed to possess the compass of the beetle; **the central complex** (*Figure 17*).

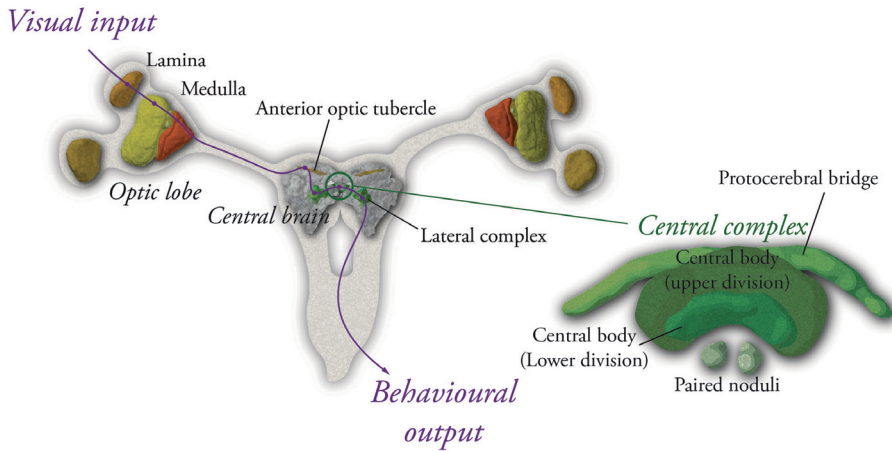


Figure 17. The light compass pathway in the dung beetle brain. Visual information from celestial cues, such as the sun or polarised skylight, first reaches the compound eye retina. The neural signals from the retinula cells are then transferred to the first processing centre for visual input; *the optic lobe*. From here, the information is transferred to the *anterior optic tubercle* and subsequently carried via the *lateral complex* to the *central complex*. This is where we find the heading direction network of the beetle. The central complex (here represented in green) is comprised of the protocerebral bridge, the upper and lower divisions of the central body and the paired noduli. Adapted from Immonen et al. 2017 (3D model of the brain was generated using the website www.insectbrain.org).

The composition of the central complex

The central complex (CX) can be divided into four neuropils, located across the midline of the insect brain: *the arch-like protocerebral bridge* (PB); *the upper division of the central body* (CBU, also termed fan-shaped body in flies); *the lower division of the central body* (CBL, also termed ellipsoid body in flies) and *the paired noduli* (NO) (Figure 17). These four regions can further be divided into slices and layers (Homberg et al., 2011; Immonen et al., 2017; el Jundi et al., 2018; Pfeiffer and Homberg, 2013), creating a highly organized neuronal network that provides connections between the individual components of the CX, as well as input from and output to other areas in the insect brain. Together, these connections transform visual information carried from the insect retina into premotor commands that guide navigation. This central region of the brain is believed to play a key role in spatial orientation (Homberg et al., 2011; Triphan et al., 2010; Varga et al., 2017; Zittrell et al., 2020), sky compass integration (Heinze and Homberg, 2007; Heinze et al., 2013; Homberg, 2004; Homberg et al., 2011; Pegel et

al., 2018) and locomotor control (Martin et al., 2015; Ritzmann et al., 2012; Seelig and Jayaraman, 2015; Strauss, 2002).

While all celestial information is encoded in the central complex, the dorsally located protocerebral bridge is considered to carry a map-like representation of the polarised skylight. In locusts, polarisation sensitive neurons (often called POL neurons) within each slice are maximally activated by a certain e-vector direction and maximally inhibited by the 90° rotation of this e-vector direction. This polarisation opponency for a preferred e-vector orientation changes by around 20-30° for each neighbouring slice (Heinze and Homberg, 2007). Considering that the same neurons are sensitive to polarised light in the dung beetle brain (el Jundi et al. 2015*), this map likely also exists in dung beetles. As there are 8 slices in the protocerebral bridge of the dung beetle CX (Immonen et al., 2017), each hemisphere of the PB covers an angular range of 180°, covering the full span of the celestial e-vector map (*Figure 10a*).

Differing neural sensitivity to polarised and unpolarised light influences the orientation strategy of the beetle

To understand how the integration of celestial compass information is encoded, and how this information is weighted in the dung beetle compass, diurnal and nocturnal beetles were presented with a polarised light source in combination with an unpolarised light source, of brighter or lower light intensities (corresponding to sun -or moonlight conditions), while simultaneously recording from the CX of the beetle (el Jundi et al., 2015a*). When presented with either of the two intensity paradigms, the CX neurons of the diurnal *Kheper lamarcki* would always respond exclusively to the unpolarised light source. In contrast, the CX neurons of the nocturnal *Scarabaeus satyrus* would only respond to the unpolarised light source during bright light conditions. If presented with dim light conditions, the neural tuning of the CX neurons in the nocturnal species would be dominated by the polarisation stimulus. This neural response, elegantly matches the relative weighting strategy of both species during straight-line orientation: while the diurnal *K. lamarcki*, known to predominantly weight directional information from the sun (Byrne et al., 2003; Dacke et al., 2014), continues to orient to the moon at night (el Jundi et al., 2015a*), its nocturnal counterpart, *S. satyrus*, will shift its predominant weight of directional information, from polarised light at night (Dacke et al., 2004; Dacke et al., 2011; Foster et al., 2019*), to the sun during the day (el Jundi et al., 2015a*). In locusts, the absolute sensitivity of the CX neurons to polarised light is higher than to unpolarised light. Thus, depending on the light intensities presented to the animal, either polarisation input or unpolarised light input will dominate the response of the neurons (Kinoshita et al., 2007). The same is suggested to happen in the compass of the beetle; at dim-light intensities, the neural response is in favour of the polarised light source. However, this only applies for as long as the eye is sensitive enough to detect the signal. A larger dorsal rim area, such as in the crepuscular *S.*

zambesianus (Dacke et al., 2003) or the diurnal homing beetle *Pachysoma striatum* (Dacke et al., 2002), might also be found in *S. satyrus* (Dacke, *unpublished*). This can, at least partially, contribute to a higher sensitivity to polarised light, influencing the weight relationship between these two sources of information even at earlier stages in the brain. In the case of the diurnal *K. lamarcki*, the dim polarised light was perhaps simply undetectable due to its very limited dorsal rim area (see *The dorsal rim area detects polarised light*), resulting in no (or very weak) neural response for dim polarised light.

The results seen in the nocturnal *S. satyrus* might also explain the weighting strategy seen behaviourally in the diurnal woodland-living beetle, *Sisyphus fasciculatus*, studied in **Paper II** and **Paper III**. As previously mentioned, this beetle attributes higher relative weight to celestial polarised light, despite the sun being fully visible (*Figure 8a, graph 1*). It is feasible that the eye of *S. fasciculatus* has a substantial dorsal rim area (*Figure 16b*). However, at this time, this is merely a speculation.



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Paper I



Research



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The effect of step size on straight-line orientation

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Moving along a straight path is a surprisingly difficult task. This is because, with each ensuing step, noise is generated in the motor and sensory systems, causing the animal to deviate from its intended route. When relying solely on internal sensory information to correct for this noise, the directional error generated with each stride accumulates, ultimately leading to a curved path. In contrast, external compass cues effectively allow the animal to correct for errors in its bearing. Here, we studied straight-line orientation in two different sized dung beetles. This allowed us to characterize and model the size of the directional error generated with each step, in the absence of external visual compass cues (*motor error*) as well as in the presence of these cues (*compass* and *motor errors*). In addition, we model how dung beetles balance the influence of internal and external orientation cues as they orient along straight paths under the open sky. We conclude that the directional error that unavoidably accumulates as the beetle travels is inversely proportional to the step size of the insect, and that both beetle species weigh the two sources of directional information in a similar fashion.

1. Background

To successfully locate mates, find food or escape predators or unfavourable environments, most animals need to travel along a given bearing in a relevant direction. For this, two main sources of directional information can be used; (i) information given by internal (proprioceptive) cues, for instance, by body rotations or leg movements [1–3], and (ii) information derived from external compass cues, such as the sun or the earth's magnetic field [4–8]. In practice, if an animal relies on internal mechanosensory information alone, it is not able to travel any greater distance from its current position (following a Brownian search where the distance travelled is proportional to the square root of the number of steps taken). This is clearly modelled by Cheung *et al.* [9]: an agent moving forward using only proprioceptive cues will, to some extent, with each successive step, deviate from the angular direction of the previous step taken. This is due to the accumulation of noise in the motor and sensory systems that will unavoidably result in the loss of ability to maintain the desired direction, subsequently making straight-line orientation impossible. This has been demonstrated behaviourally in animals as diverse as humans, spiders and beetles [10–12].

In most navigating and migrating animals, directional guidance is acquired from multiple sources of information [13–16], originating from the movement of the animal itself (*internal cues*) [1–3] and/or from its surroundings (*external cues*) [17–19]. Experimental studies from species as diverse as ants [16], butterflies

[20], elk [21] and grey seals [22] have considered how animals may balance these two sources of directional information to navigate within their local environment. In ants, different sources of directional information are weighted relative to their respective certainty, where the cue conveying the highest certainty is afforded the highest weight in the directional output from the compass [16,23,24]. For instance, if the visual scenery fails to provide the information needed, the ant will rely more strongly on the directional information provided by its path integrator to complete its navigational task [16].

In contrast to most migrating and homing animals, a ball-rolling dung beetle simply needs to move along one single bearing for the duration of its current travels. At the dung pat—where the journey of a ball-rolling beetle begins—competition for dung can be fierce [25]. In order to obtain a sufficient amount of food, beetles gather and shape dung into balls and roll them away. To ensure the most efficient escape from the competitors at the pat, the beetles exit in all directions along paths as straight as the terrain allows [6,26–29]. In this way, with every step taken, they maximize the distance between themselves and their competitors. For diurnal beetles, the most prominent compass cue used to steer along this set bearing is the sun [6,27,28,30]. Together with the celestial polarization pattern, the skylight intensity gradient and the colour gradient across the sky, this forms a highly robust straight-line orientation system [30–32]. If these cues are eliminated from the ball-rolling dung beetle's field of view, the animal soon loses its ability to maintain a straight bearing [12].

Understanding how animals balance internal and external directional cues to maintain a straight bearing is still an open question in movement ecology. It is challenging to model behaviour in the ecological context of homing insects [16,20], elks [21] and seals [22] that forage within a familiar territory. This is because most theoretical models assume a straight-line optimal trajectory [33], while the actual trajectories travelled by these animals may be more tortuous. In contrast, ball-rolling dung beetles strive to move along straight paths from the start to the end of their journeys [6,12,27–30]. Here, we characterize the size of the directional error generated with each step in the presence or absence of external compass cues in two closely related species of dung beetle, that differ greatly in size. This allows us to (i) estimate the influence of step size on the precision of straight-line orientation [34,35] and (ii) model the weight given to external sky compass cues over internal proprioceptive cues for straight-line orientation in dung beetles.

2. Methods

2.1. General

Two closely related species of diurnal dung beetles, *Scarabaeus (Scarabaeus) ambiguus*, and *Scarabaeus (Kheper) lamarcki* were collected within the elephant park Adventures with Elephants (27.95°E, 24.78°S) and Stonehenge game farm (24.32°E, 26.39°S), respectively, in South Africa with the aid of dung-baited pitfall traps. Experiments were performed at Stonehenge game farm and Thornwood lodge (28.02°E, 24.77°S), during February 2017. All experiments (apart from the studies of orientation performance in the absence of visual cues which were conducted in complete darkness in a light-tight indoor laboratory) were performed outdoors under clear skies at solar elevations between 45 and 60°. Each beetle was marked individually with a number on

its thorax using a white marker (Tipp-Ex®). An overhead Sony Handycam HDR-CX730E (fitted with a 0.42× wide angle lens to extend the field of view when required) was used to record dung beetle rolling trajectories and exit bearings.

2.2. Step size determination

Individual beetles were allowed to roll their dung ball across a flat, sand-coated (Dried Ochre, granular paint, Fired Earth™), 50 cm radius, outdoor arena (figure 1a). Step size was determined as the distance from the point at which the front foreleg (left or right) was stable on the arena surface, to the point at which the same limb was again stable on the surface. Image processing software, ImageJ1© (National Institutes of Health, Bethesda, MD, USA), was used to extract the x and y coordinates of the start and endpoint of each step from the overhead videos. From this, the length of the step was calculated and converted to true length by using a millimetre scale present in the frame for reference. The step length for each species was determined by calculating the average length of 10 strides per beetle for each species ($N = 10$).

2.3. The role of the sun in the celestial compass system of the two beetle species

Beetles were placed individually alongside their dung ball in the centre of a flat, circular, sand-coated, wooden, outdoor arena, measuring 75 cm in radius, and allowed to roll the ball to the edge, where the exit bearing was noted. The beetle was then removed from its ball and placed back in the centre of the arena alongside its ball. At the same time, the sun's apparent position was changed by 180°, using a mirror (30 × 30 cm), while simultaneously concealing the real sun from the beetle's field of view with a wooden board (100 × 75 cm). Again, the beetle was allowed to roll to the edge of the arena and its second exit bearing was noted. A third trial, with an un-manipulated sun position as in the first trial, was performed as a control to verify that the beetle strived to adhere to approximately the same bearing throughout the experiment. This held true for all beetles tested. In total, 45 individuals per species rolled from the centre to the edge of the arena three times.

Angular change was calculated as the difference in exit bearing between two exits from the arena (figure 2). Directional statistics were obtained from Oriana 3.0 (Kovach Computing Services, Anglesey, UK).

2.4. The effect of step size on orientation performance

To eliminate any influence of size or shape of beetle-made dung balls on the orientation performance of the different beetles, 'standard balls' were made from dung infused Play-Doh® (Hasbro, Pawtucket, RI, USA). These balls had a set size of 1.7 cm diameter for *Scarabaeus ambiguus* and 3.5 cm diameter for *S. lamarcki* (figure 1a). These dimensions were determined from the average diameter of beetle crafted balls for each species (*S. ambiguus*: 1.74 ± 0.13 cm (mean \pm s.e.m.), *S. lamarcki*: 3.54 ± 0.60 cm) ($N = 10$).

2.4.1. The effect of step size on orientation performance in the absence of external visual cues

In these experiments, beetles were individually placed beside a 'standard ball' of the size associated with their species (figure 1b), at the centre of a flat, circular, sand-coated, wooden, arena, measuring 50 cm in radius in the complete darkness. From here, the beetle rolled the ball to the perimeter of the arena. This marked the end of the trial (figure 3). To record the beetles' trajectories in the dark, the overhead camera was fitted with an infrared lamp, and individuals were marked with high-gain reflective

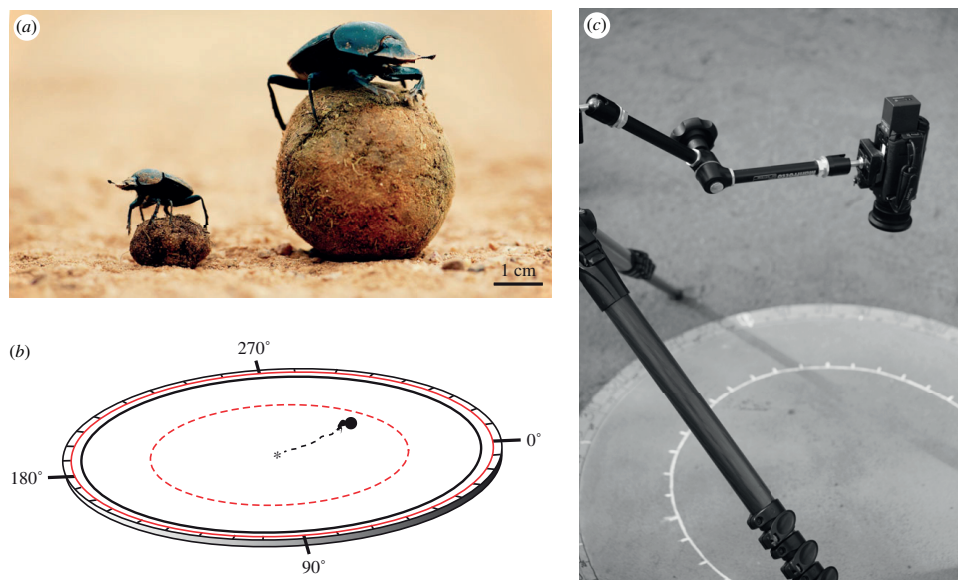


Figure 1. Description of the experimental arenas and beetles. Individuals of *Scarabaeus ambiguaus* (left) and *S. lamarki* (right) are depicted side-by-side for size comparison. Photo: Christopher Collingridge (a). For all treatments, a beetle was placed alongside a ball in the centre of a circular, sand-coated arena (b) and filmed with an overhead camera (c). The beetle was allowed to roll its ball to the perimeter of the arena, where the exit angle was noted (0° = magnetic North). Depending on the experimental treatment, the beetle either rolled only once, or was repeatedly placed back in the arena centre beside its ball until it had exited the arena 20 times. The ball rolled was either a natural dung ball or a standard putty ball (shown in a). For the experiment where the sun was mirrored, a 75 cm radius arena was used (not depicted in the figure). For all other experiments conducted, three differently sized arenas were used depending on the species of beetle (b): 50 cm (*S. ambiguaus* and *S. lamarki*, black solid line), 33 cm (*S. ambiguaus*, red dotted inner circle), 52 cm (*S. lamarki*, red solid outer circle). (Online version in colour.)

paint (Soppec©: Technima Nordic AB, Mölndal, Sweden) on their thorax. In total, 10 individuals per species were tested.

The beetle's position in each video frame was determined using custom-made tracking software in Matlab R2016a (Mathworks Inc., Natwick, MA, USA, courtesy of Dr Jochen Smolka). Camera calibration software in Matlab was used to correct for optical distortion and true distances were obtained from a calibration pattern (3×3 cm black and white squares) placed on the surface of the arena. Path length of each roll was calculated by summing the two-dimensional distance travelled between successive frames. To determine how straight the beetle's trajectories were, a *straightness index* (S) was calculated as D/W [36], where D is the distance from the starting point to the perimeter of the arena, and W is the length of the path taken.

In order to determine the angular error generated by each step in the absence of external visual cues, 15 individuals of each species were filmed at close range, with an overhead camera fitted with an infrared lamp. Using a custom-made tracking software in Matlab 2017b (Mathworks Inc. Natwick, MA, USA), the angular error generated by each step of the beetle, was determined. For this, we defined a step as the instance of foreleg-surface contact. The position of two consecutive surface contacts by the same foreleg was tracked and a vector between these two consecutive points was drawn to determine the bearing direction of one step. From this, angular error per step was calculated as the absolute difference in bearing direction between two consecutive vectors (figure 4a).

2.4.2. The effect of step size on orientation performance in the presence of external visual cues

To determine the orientation performance of the two beetle species under an open sky, each beetle, together with a species

specific 'standard ball', was repeatedly placed in the centre of a flat, circular, sand-coated, wooden, outdoor arena, until each beetle had rolled its ball to the edge of the arena 20 times. Two different sized arenas were used (figure 1c); (i) one with the effective radius set to a distance equivalent to the length of 20 steps for the species tested (*S. ambiguaus*: 32.38 cm, *S. lamarki*: 51.79 cm) and (ii) one with a radius of 50 cm. The exit bearings of 20 rolls performed by each beetle were again noted and all trajectories were recorded from above. In total, 20 individuals per species were tested.

Orientation performance of each individual was determined by the mean resultant vector length (R) calculated in Oriana 3.0 (Kovach Computing Services, Anglesey, UK) from the 20 exit bearings for one individual (figure 5). This value is used to describe the concentration of unimodal circular distribution, and ranges from a value of 0 (random distribution of angles) to a value of 1 (no dispersion in distribution of angles). The better oriented the beetle, the closer the exit bearings cluster around one direction, and the closer the mean resultant vector is to unity. In total, the orientation performance of 20 individuals per species for the treatments described above were recorded.

2.5. Estimation of motor errors, compass errors, and their weight

The integration of proprioceptive cues in the orientation system of an agent generates noise (termed *motor error*) affecting the motoric output of the agent. Similarly, the detection and integration of external compass cues by the orientation system generates noise (termed *compass error*). Both of these sources of noise can be expected to affect the motoric output of an agent exercising straight-line orientation.

The *biased correlated random walk model* [35] was used to estimate the compass error of external cues and determine how much weight is given to external visual cues over internal proprioceptive cues for straight-line orientation in the beetles. In both *biased* and *correlated random walks*, the agent's goal is to walk in a straight line in an arbitrary direction θ_0 (figure 4a). In a *biased random walk* (BRW) the instantaneous angular error, θ_v , arises from noise in external visual cue acquisition (*compass error*), however, in a *correlated random walk* (CRW) it arises from accumulated noise in motoric execution (*motor error*). The *biased correlated random walk model* combines the two errors in the following manner:

$$\Delta X_{i+1} = l[w \cos(\theta_0 + \theta_i^{\text{BRW}}) + (1 - w) \cos(\theta_i + \theta_i^{\text{CRW}})] \quad (2.1)$$

and

$$\Delta Y_{i+1} = l[w \sin(\theta_0 + \theta_i^{\text{BRW}}) + (1 - w) \sin(\theta_i + \theta_i^{\text{CRW}})] \quad (2.2)$$

where l is the step length of the current step, θ_i is the direction of movement of the previous step, θ_i^{CRW} is a motor error term, θ_i^{BRW} is a compass error term, and $w \in [0, 1]$ is the weighting given to external cues. We assume that θ_i^{CRW} and θ_i^{BRW} are random angles drawn from a von Mises distribution with a zero-mean and standard deviation θ^{CRW} and θ^{BRW} , respectively. Table 1 summarizes the model parameters and their experimental equivalents. θ^{CRW} was estimated for each species from the angular errors experimentally measured in the absence of visual cues and used as input parameters to the model (figure 4b, step 1). BCRW trajectories with the estimated value of θ^{CRW} were generated numerically ($N = 500$) with a range of values for w and θ^{BRW} (figure 4b, step 2). The resulting mean vector length, R , was compared against the experimentally measured R (figure 4b, step 3) to estimate pairs of w and θ^{BRW} for the two different sized beetle species (figure 4b, step 4). Due to rotational symmetry, R is independent of the direction θ_0 , hence we arbitrarily set its value to zero.

2.6. The role of step size in the natural habitat

Ten individuals of each species were allowed to sculp and roll a dung ball from a pat of 11 cow dung, placed in the savannah on a sunlit day (figure 6). Trials alternated between the two species. The paths of the beetles were recorded by a hand-held video camera (GoPro® HERO5 Black), held approximately 1 m above the beetle as it rolled, until the beetle started to bury its ball. Bearing direction and distance from the centre of the dung pat to the site of burial were then measured. Positional information for the individual segments of the path trajectories from beetles rolling across this natural terrain was obtained using a tailor-made analysis tool [37] (courtesy of Dr Benjamin Risse, University of Münster). To extract shape and total distance travelled for each trajectory, a custom-made Matlab script was used.

3. Results

3.1. Body size and step size determination

The two closely related, ball-rolling species of South African dung beetles, *Scarabaeus ambiguus* and *S. lamarcki* differ significantly in size with a pronotum width of 1.09 ± 0.01 cm and 2.07 ± 0.03 cm and a body length (tip of abdomen to tip of pronotum) of 1.52 ± 0.01 cm and 2.86 ± 0.04 cm (mean \pm s.e.m. $N = 10$) respectively (Wilcoxon rank-sum test; $p_{\text{Pronotum}} < 0.001$, $p_{\text{Body length}} < 0.001$, $Z_{\text{Pronotum}} = -3.75$, $Z_{\text{Body length}} = -3.76$). Not surprisingly, the average step size for the smaller *S. ambiguus* (1.69 ± 0.09 cm, $N = 20$), is significantly shorter than that of the larger *S. lamarcki* (2.89 ± 0.08 cm, $N = 20$) (Wilcoxon rank-sum test; $p < 0.001$).

3.2. The role of the sun in the celestial compass system of the two beetle species

To investigate the role of the sun in the compass system of the two species, the response of an orienting beetle in terms of change in roll bearing, was tested under an un-manipulated sky as well as under a sky with the position of the sun changed by 180° by the use of a mirror (and the real sun simultaneously shielded from view of the beetle). When the position of the sun was artificially changed by 180° (*test*) on the second roll, both species changed their headings in response to this manipulation, with the same order of magnitude ($\mu_{S. ambiguus} = 152.37^\circ \pm 105.29^\circ$, $\mu_{S. lamarcki} = 139.39^\circ \pm 117.45^\circ$, Mardia–Watson–Wheeler test; $p = 0.92$, $N = 45$, $W = 0.16$) (figure 2). No significant change in direction (μ) was seen in individuals of either of the two species of beetles between two rolls made under an unobscured sky (*control*) ($\mu_{S. ambiguus} = -3.61^\circ \pm 53.56^\circ$ (mean \pm s.d.), $\mu_{S. lamarcki} = 4.43^\circ \pm 38.61^\circ$, V -test (with the expected mean of 0°); $p_{S. ambiguus} < 0.001$, $p_{S. lamarcki} < 0.001$, $N = 45$, $V_{S. ambiguus} = 0.65$, $V_{S. lamarcki} = 0.79$). This demonstrates that both species orient using a sun compass.

3.3. Orientation performance in the absence and presence of external visual cues

3.3.1. In the absence of visual cues

When rolling over a radial distance equivalent to the length of 20 steps of the respective species (*S. ambiguus*: 32.38 cm, *S. lamarcki*: 51.79 cm, see Methods, figure 1) there was no significant difference between the straightness of the trajectories travelled by the two species ($S_{S. ambiguus} = 0.48 \pm 0.12$; $S_{S. lamarcki} = 0.62 \pm 0.16$, Wilcoxon rank-sum test; $p = 0.08$, $Z = -0.79$) (figure 3). When instead analysing the straightness of tracks across a radial distance of 50 cm, the smaller species, *Scarabaeus ambiguus* had a lower straightness index (S) compared to the larger *S. lamarcki* ($S_{S. ambiguus} = 0.45 \pm 0.12$; $S_{S. lamarcki} = 0.65 \pm 0.17$ (mean \pm s.e.m., $N = 15$), Wilcoxon rank-sum test; $p = 0.02$, $Z = 2.32$) (figure 3).

This indicates that the size of the angular error generated by each step, θ^{CRW} , does not differ between the two species of beetles. This was further confirmed from the direct comparison between species (*S. ambiguus*: $33.11^\circ \pm 5.12^\circ$, $N = 5$; *S. lamarcki*: $29.41^\circ \pm 9.92^\circ$, $N = 10$, Wilcoxon rank-sum test; $p = 0.86$, $U = 42$).

3.3.2. In the presence of visual cues

When rolling 20 times across an arena with a radius proportional to 20 step lengths of the two species (*S. ambiguus*: 32.38 cm, *S. lamarcki*: 51.79 cm), we found no significant difference in mean resultant vector length (i.e. spread of exit bearings) between the different sized beetles ($R_{S. ambiguus} = 0.91 \pm 0.015$; $R_{S. lamarcki} = 0.91 \pm 0.02$, Wilcoxon rank-sum test; $p = 0.42$, $N = 20$, $Z = -0.83$) (figure 5). When instead testing the orientation performance over a radius of 50 cm, *Scarabaeus ambiguus* showed a significantly shorter mean resultant vector length compared to that of *S. lamarcki* ($R_{S. ambiguus} = 0.88 \pm 0.02$; $R_{S. lamarcki} = 0.92 \pm 0.01$, Wilcoxon rank-sum test; $p = 0.028$, $N = 20$, $Z = -2.20$) (figure 5). This indicates that *S. ambiguus*, the smaller of the two species, is less able to maintain its bearing over a given distance compared to its larger relative when rolling under a natural sky.

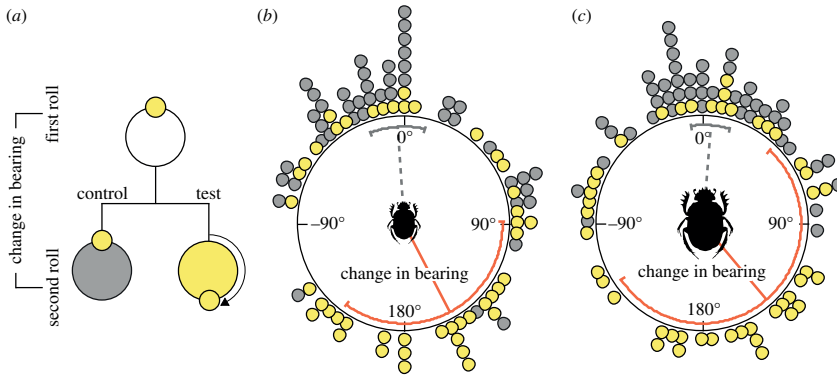


Figure 2. The role of the sun in the celestial compass system of two beetle species. The response to a mirrored sun while rolling outdoors, under a clear sky, was tested in two closely related, but differently sized beetle species. A schematic diagram of the experiment is presented in (a). Forty-five individuals of *Scarabaeus ambiguus* (b) and *S. lamarcki* (c), respectively, were individually placed under the natural sky, alongside a dung ball in the centre of a 75 cm radius, circular sand-covered arena. The beetles were allowed to roll to the perimeter of the arena where their exit angles were noted. From here, the beetles were placed back in the centre to exit a second time, either under (i) the same natural sky as during the previous roll (*control*, grey circles), or (ii) a manipulated sky where the apparent position of the sun is changed by 180° by the use of a mirror (*test*, yellow circles). The difference between two exit angles was calculated and used to define the mean change in bearing (*control*, dotted grey lines; *test*, solid red lines). Error bars represent one circular standard deviation. When allowed to roll twice under the sun, individuals of both species showed no significant change in bearing between consecutive rolls (dotted grey line). Under the mirrored sun, both species responded to this treatment by a change in exit bearing approaching 180° (solid red lines). (Online version in colour.)

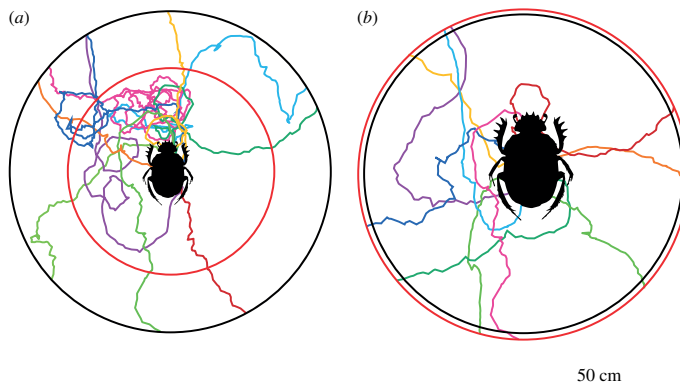


Figure 3. Rolling trajectories in the absence of external visual cues. The two closely related, but differently sized beetles, were allowed to roll a dung ball from the centre of a flat, sand-coated arena, in complete darkness. The full trajectories of 10 randomly chosen beetles of each species are shown. On the 50 cm radius arena (black perimeter) *S. ambiguus* (a), obtained a significantly lower *straightness index* (higher tortuosity) compared to the larger *S. lamarcki* ($p = 0.02$, $N = 15$) (b). When analysed over a radial distance corresponding to 20 steps for each species respectively (32 cm for *S. ambiguus* and 52 cm for *S. lamarcki*) (a, inner red perimeter; b, outer red perimeter) no significant difference in straightness was recorded ($p = 0.08$, $N = 15$). (Online version in colour.)

We found no difference in the straightness of the 20th exit path compared to the 1st exit path performed by the same individual (Wilcoxon rank-sum test; $p_{50 \text{ cm}(S. \text{ ambiguus})} = 0.16$, $Z = 1.42$; $p_{50 \text{ cm}(S. \text{ lamarcki})} = 0.16$, $Z = 1.42$; $p_{\text{step length}(S. \text{ ambiguus})} = 0.30$, $Z = 1.04$; $p_{\text{step length}(S. \text{ lamarcki})} = 0.16$, $Z = 1.42$, $N = 20$). This suggests that the error generated with each step does not change in size with distance rolled but remains the same regardless of the number of steps taken.

3.4. Weighting of compass and motor errors

The angular error generated by each step in the absence of external visual compass cues was introduced as an estimation

of motor error (θ^{CRW}) in the *biased correlated random walk model* [36] (figure 4b, step 1). This allowed us to compare the resulting mean vector length, R , of the modelled data against the experimentally obtained R values (figure 4b, step 3) to estimate pairs of w (the balance between CRW and BRW) and σ^{BRW} (standard deviation of compass error) for the two species (figure 4b, step 4). From this, no significant difference was found in the balance between CRW and BRW, between the two species ($w_{S. \text{ ambiguus}}: 0.84 \pm 0.09$, $w_{S. \text{ lamarcki}}: 0.83 \pm 0.08$; Wilcoxon rank-sum test; $p = 0.54$, $N = 13$, $U = 183$). This also held true for the mean compass error σ^{BRW} ($\theta_{S. \text{ ambiguus}}^{\text{BRW}}: 5.95^\circ \pm 2.97^\circ$, $\theta_{S. \text{ lamarcki}}^{\text{BRW}}: 6.34^\circ \pm 3.01^\circ$,

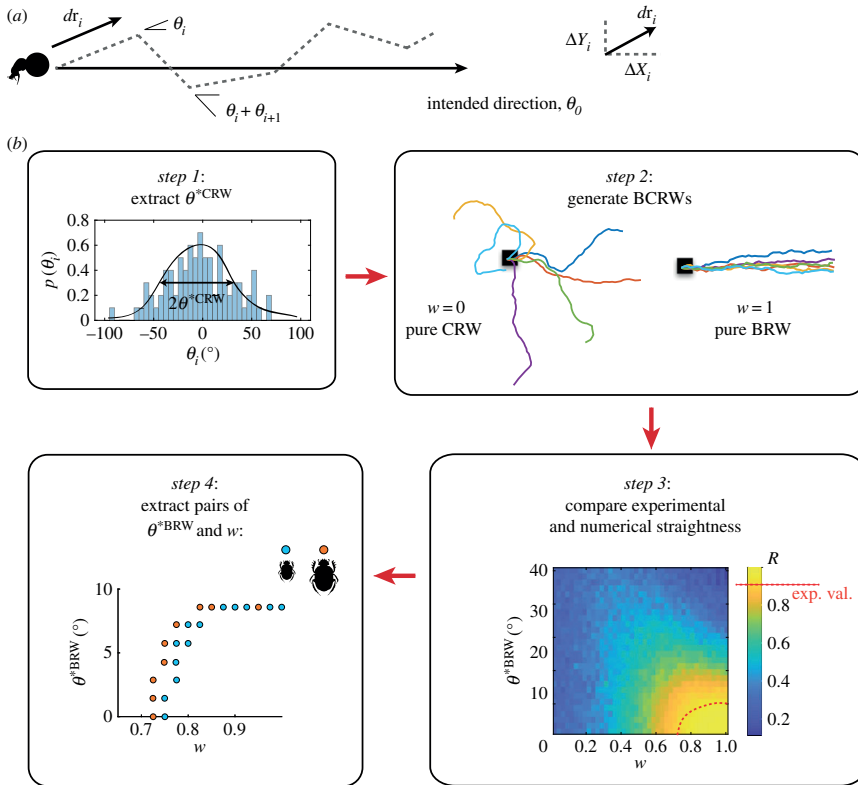


Figure 4. Estimation of motor errors, compass errors, and their balance. A model of a beetle performing a random walk where θ_i is the direction of movement of the previous step and ΔX_i , ΔY_i are the distance travelled in step i along the x and y directions, respectively (defined in equations (2.1) and (2.2)) (a). A flow diagram describing the process of estimating pairs of w and θ^{BRW} for two beetle species that differ in size (b). *Step 1:* θ^{CRW} is estimated from the width of the angular errors of a beetle orienting in the absence of visual cues. *Step 2:* Two sets of BCRW trajectories are illustrated; one at the limit of pure CRW ($w=0$) and one at the limit of pure BRW ($w=1$). To generate these trajectory examples, we chose arbitrarily $\theta^{\text{BRW}} = \theta^{\text{CRW}} = 5^\circ$ (these values were arbitrarily chosen for the purpose of illustrating the model). Each trajectory is shown in a different colour. *Step 3:* Mean vector length (R) for each species is generated from the simulation and compared to the experimentally measured values (shown as red dotted line on the colour bar and on the heat-map). *Step 4:* The extracted w and θ^{BRW} for each species is shown. (Online version in colour.)

Wilcoxon rank-sum test; $p=0.65$, $N=13$, $U=205.5$). This suggests that the relative balance between internal and external compass cues for straight-line orientation in beetles is not affected by differences in stride length. This finding is consistent with the model hypothesis.

3.5. Orientation performance in the natural habitat

When allowed to form a ball and roll it away from a dung pat under a clear, sunlit sky in their natural habitat (figure 6a), the smaller species, *Scarabaeus ambiguus*, buried their dung balls $7.56 \text{ m} \pm 1.05 \text{ m}$ ($N=10$) away from the pat. This is significantly closer to the pat than the average radial distance travelled by the larger species before burial (*S. lamarki*: $12.45 \text{ m} \pm 1.28 \text{ m}$, $N=10$) (Wilcoxon rank-sum test; $p=0.010$, $Z=2.57$) (figure 6b). Interestingly, the average total distances rolled to reach these burial spots did not differ between the two species (*S. ambiguus*: $20.43 \text{ m} \pm 4.54 \text{ m}$, $N=10$, *S. lamarki*: $18.66 \text{ m} \pm 1.94 \text{ m}$, $N=10$) (Wilcoxon rank-sum test; $p=0.85$, $Z=0.19$). This suggests that there is a behavioural mechanism

to compensate for the increase in tortuosity that the smaller beetles unavoidably seem to experience (figure 6).

4. Discussion

4.1. Maintaining a direction in the absence of external visual cues

When deprived of all visually mediated compass cues, the dung beetles failed to maintain a straightforward course and instead moved along tortuous paths (figure 3). Unsurprisingly, spiders, amphibians and humans are also unable to move along a given bearing in the dark [10,38,39]. Under these circumstances, these animals are expected to travel by means of a *correlated random walk* (CRW) [33], where each step is intended to point in the same direction as the previous one. This is also the case for the dung beetle, as captured by the *biased correlated random walk model* in the limit $w=0$ (i.e. with no external compass input, and thus only the

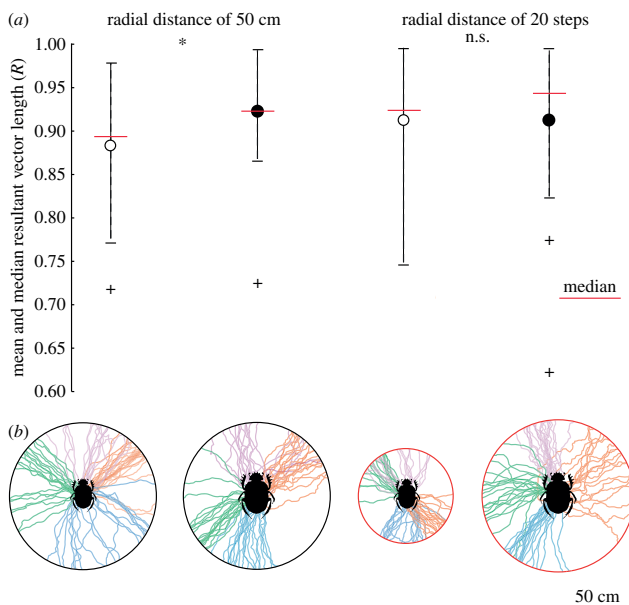


Figure 5. Measuring orientation performance in the presence of external visual cues. As a measure of orientation performance (a), the mean vector length for each beetle was calculated from 20 tracks over a radius equivalent of 50 cm, as well as of a radius equivalent of 20 step lengths of the corresponding species (32 cm for *Scarabaeus ambiguus* and 52 cm for *S. lamarcki*) (white circle, mean value for *S. ambiguus*; black circle, mean value for *S. lamarcki*; red solid line, median value for *S. ambiguus* and *S. lamarcki*). An R -value of 1 indicates that the beetles maintained the same direction over 20 rolls. When rolling over a radius of 50 cm, the smaller species, *S. ambiguus* ($N = 20$), showed a significantly shorter resultant vector length compared to the larger species ($N = 20$) ($R_{S. ambiguus}$: 0.88 ± 0.02 ; $R_{S. lamarcki}$: 0.92 ± 0.01 , $p = 0.028$, $N = 20$). However, no significant difference was seen when both species rolled over a distance equivalent to 20 steps ($R_{S. ambiguus}$: 0.91 ± 0.015 ; $R_{S. lamarcki}$: 0.91 ± 0.02 , $p = 0.42$, $N = 20$). Paths travelled by four individuals for each species and radial distance (b) are shown (from left: *S. ambiguus* (50 cm); *S. lamarcki* (50 cm); *S. ambiguus* (32 cm); *S. lamarcki* (52 cm)). Each colour represents 20 trajectories of one individual. $* = p < 0.05$, $n.s. = p > 0.05$. There was no difference in the straightness of the 20th exit path compared to the 1st exit path performed by the same individual in any of the conditions ($p_{50 \text{ cm}(S. ambiguus)} = 0.16$, $p_{50 \text{ cm}(S. lamarcki)} = 0.16$; $p_{\text{step length}(S. ambiguus)} = 0.30$, $p_{\text{step length}(S. lamarcki)} = 0.16$, $N = 20$). (Online version in colour.)

Table 1. Biased correlated random walk model parameters.

parameter	meaning	experimental value	theoretical value (model inputs/outputs)
l	step size	extracted from step analysis of beetles in the presence of external visual cues	set by the experimental values <i>S. ambiguus</i> : $l = 1.6$ cm <i>S. lamarcki</i> : $l = 2.6$ cm
θ_0	intended direction	extracted as mean exit bearing	0 (towards the right)
w	balance between CRW and BRW	unknown	extracted to fit properties of experimental trajectories (R):
θ^{BRW}	standard deviation of compass error	unknown	<i>S. ambiguus</i> : $\theta^{\text{BRW}} = 5.95^\circ \pm 2.97^\circ$ $w = 0.84 \pm 0.09$ <i>S. lamarcki</i> : $\theta^{\text{BRW}} = 6.34^\circ \pm 3.01^\circ$ $w = 0.83 \pm 0.08$
θ^{CRW}	standard deviation of motor error	extracted from step analysis of beetles in the absence of external orientation cues	set by the experimental value <i>S. ambiguus</i> : $\theta^{\text{CRW}} = 33.11^\circ \pm 5.12^\circ$ [$N = 5$] <i>S. lamarcki</i> : $\theta^{\text{CRW}} = 29.41^\circ \pm 9.92^\circ$ [$N = 10$]

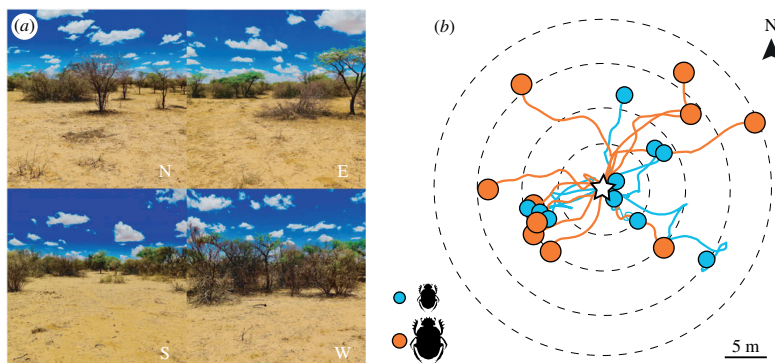


Figure 6. Orientation performance in a natural environment. The smaller *Scarabaeus ambigua* and the larger *S. lamarcki* were allowed to form a dung ball and roll it away from a dung pat (marked with a star) in their natural environment (N, north; E, east; S, south; W, west) (a). Their trajectories (blue lines, *S. ambigua*; orange lines, *S. lamarcki*), were recorded until they started to bury their balls (blue circles, *S. ambigua*; orange circles, *S. lamarcki*) (b). This marked the end of the trial and the radial distance from the dung pat to the site of burial was measured. The dotted concentric circles indicate radial distances from the dung pat in 5 m increments. In total, trajectories of 10 individuals per species were recorded. The smaller species, *S. ambigua*, rolled a significantly shorter radial distance from the pat before burying its ball when compared to the larger species (*S. ambigua*: $7.56 \text{ m} \pm 1.05 \text{ m}$, *S. lamarcki*: $12.45 \text{ m} \pm 1.28 \text{ m}$, $N = 10$) ($p = 0.001$, $N = 10$). (Online version in colour.)

second term in equations (2.1) and (2.2) contributes to the accumulated error). Assuming that only internal sensory information was available to these beetles when orienting in the dark, a directional error, most likely caused by mechanosensory noise in the muscles of their moving limbs, accumulated with every step taken. Thus, the instantaneous angular error arising from the accumulated noise in motoric execution is determined as equivalent to the *motor error*. A direct analysis of the direction of each subsequent step when rolling in complete darkness further reveals that this motor error lies at around 30° per step, irrespective of species (*S. ambigua*: $33.11^\circ \pm 5.12^\circ$, *S. lamarcki*: $29.41^\circ \pm 9.92^\circ$). As can be expected, these findings showed that over the same radial distance (50 cm) the smaller *S. ambigua* (with more steps taken per distance travelled) rolled its ball along a significantly more tortuous trajectory compared to the larger, *S. lamarcki* (figure 3). Tortuous paths can also be observed outdoors, under overcast skies, or when the beetle is prevented from seeing the sky by the use of a cap [12]. Under these conditions, just as in the dark, the beetle cannot access any external visual compass cues to correct for the accumulation of errors in its orientation system.

4.2. Dung beetles rely primarily on the sun for straight-line orientation

From the trajectories presented in figure 5 it is evident that, under the open sky, both species of dung beetles orient along straight paths in a given direction, presenting a clear contrast to the more tortuous trajectories taken in the absence of external visual cues (figure 3). The large change in roll bearing recorded for *Scarabaeus ambigua* and *S. lamarcki* in response to a 180° displacement of the sun (figure 2) demonstrates the common use of a sun compass in these species during straight-line orientation. These results are well in line with previous studies of the celestial compass system of *S. lamarcki* [6,27–30].

When rolling repeatedly to the edge of the 50 cm diameter arena, under an open sky, the larger *S. lamarcki* was significantly better oriented than its smaller relative *S. ambigua* (figure 5a). This difference in performance no longer prevailed when the orientation performance over a radius proportional to 20 steps of the two species (*S. ambigua*: 32.38 cm, *S. lamarcki*: 51.79 cm) was considered. This again suggests that both species gain a similar sized error with every step taken.

Interestingly, the error that is accumulated while rolling seems not to accumulate over the course of 20 consecutive rolls, as no difference in straightness was found between the first and last roll for either of the species while rolling under an open sky (figure 5). This clearly demonstrates that the error generated by each step taken, while the beetle is rolling its ball, remains the same size irrespective of the number of steps taken.

4.3. A biased correlated random walk supports straight-line orientation in dung beetles

To understand the effect of step size on straight-line orientation [34,35], and to model how much weight is given to external sky compass cues over internal proprioceptive cues for straight-line orientation in dung beetles, we chose to connect a vector-weighted *biased correlated random walk model* for directed movement, where external cues are balanced with internal ones [34,35]. The model assumes that the beetle intends to move in a straight line, which is what we have observed in this and many earlier studies of dung beetle orientation (figure 5b) [6,12,26–32].

4.4. The weighting of different cues provides the best possible compass strategy

The values for the angular error generated in the absence of visual cues, determined as equivalent to the motor error (33° for *S. ambigua* and 29° for *S. lamarcki*), were used as input parameters to the biased correlated walk model,

allowing us to estimate the balance between a *biased random walk* (BRW) and a CRW employed by a beetle when orienting outdoors. From this model, we can also describe the compass error generated with each step in the two species of dung beetles.

When the beetles are rolling outdoors, under the full view of a natural sky, the weight given to external cues over internal cues is significantly shifted towards external cues ($w_{S. ambigua} = 0.84$ and $w_{S. lamarki} = 0.83$), revealing that the paths of the beetles, irrespective of beetle size, are primarily dictated by a BRW. This balance did not differ between the species.

Interestingly, our model further reveals that the compass error is remarkably smaller than the motor error (compass error: *S. ambigua*: 5.94° and *S. lamarki*: 6.34° versus motor error: *S. ambigua*: 33.11° and *S. lamarki*: 29.41°) with no significant difference in the size of the error between the two species. This difference in angular error, or ‘noise’, generated by the two sources of information (motor and compass) provide a possible explanation for the shift towards external cues by the compass when orienting outdoors.

In parallel to the weighting of external visual cues over internal proprioceptive cues by the dung beetle, ants also seem to rely more heavily on the cue that currently provides the more precise directional information [16]. If directional information from the path integrator (PI) of the ant and the visual scene are set in conflict, the weighting towards the PI will increase as the ants move further from their nest, and their PI vector becomes increasingly longer.

In summary, our results suggest that (i) the analysis systems of the compass cues (visual system and neuronal system) of the smaller beetle are as precise as that of the larger beetles and that (ii) the compass system of the smaller beetles (as in the balance between a CRW and a BRW) is not specifically evolved to compensate for the directional challenges that arise due to differences in stride length.

4.5. The effect of step size on straight-line orientation in the natural habitat

A recent study on ants shows that the ability to orient using compass information is the same across ants that differ in size by a factor of three, but similar to this study, the accumulation of errors increases with the number of steps taken [40]. Consequently, smaller ants, just like the beetles, can be observed to move over more tortuous paths than their larger relatives. Together, these studies nicely demonstrate that the ‘step size error’ has an effect on the ability of these insects to maintain a straight bearing even under an open sky, suggesting that regardless of the availability of an external visual compass cue, this noise cannot be fully compensated for and will work to the disadvantage of the smaller species. The recorded accumulation of error with distance rolled in the

beetles is most likely partly due to mechanosensory noise in the motor system when executing each step, and partly due to noise in the acquisition and processing of the celestial compass cues that direct the steps taken [41].

Smaller insects with smaller steps risk travelling along more tortuous, and thus more energetically costly paths, compared to their larger relatives. But, this is only a disadvantage if the orienting insects—regardless of size—aim to travel the same distance. For the beetles, this is not the case. The smaller beetles tended to bury their balls closer to the dung pat, compared to the larger beetles within the same terrain (figure 6*b*). The phenomenon of smaller sized individuals travelling shorter distances than their larger peers, is not uncommon [42–45], on the contrary, there is a strong positive correlation between the distance an animal travels and its body size. In the case of the beetle, it is still unknown how it measures the distance travelled, but the impact of the relative speed at which angular errors accumulate might play a role in this behavioural outcome, resulting in smaller beetle species reaching shorter effective distances with their balls of dung. This will be the focus of future studies.

5. Conclusion

Our results show that for an orienting ball-rolling beetle, an angular error accumulates over each step in the absence as well as in the presence of external visual compass cues. Consequently, smaller insects, with proportionally shorter legs, will produce a larger directional error over the same distance travelled. Our results further imply that the nature of the compass systems of different sized insects is not specifically evolved to compensate for the size (step size) of the animal.

Ethics. All experiments in this study were performed in accordance with the regulations referred by the South African and Swedish guidelines for animal experiments.

Data accessibility. The datasets supporting this article are available as electronic supplementary material.

Authors' contributions. L.K., O.P., C.T., M.B. and M.D. conducted experiments; L.K., O.P., L.M. and M.D. designed experiments; L.K., O.P. and M.D. analysed the data; L.K. drafted the manuscript; O.P., L.M., M.B. and M.D. revised the manuscript. All authors are accountable for the presented work and approved the final version of the manuscript for publication.

Competing interests. We declare we have no competing interests.

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Paper II





Straight-line orientation in the woodland-living beetle *Sisyphus fasciculatus*

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Abstract

To transport their balls of dung along a constant bearing, diurnal savannah-living dung beetles rely primarily on the sun for compass information. However, in more cluttered environments, such as woodlands, this solitary compass cue is frequently hidden from view by surrounding vegetation. In these types of habitats, insects can, instead, rely on surrounding landmarks, the canopy pattern, or wide-field celestial cues, such as polarised skylight, for directional information. Here, we investigate the compass orientation strategy behind straight-line orientation in the diurnal woodland-living beetle *Sisyphus fasciculatus*. We found that, when manipulating the direction of polarised skylight, *Si. fasciculatus* responded to this change with a similar change in bearing. However, when the apparent position of the sun was moved, the woodland-living beetle did not change its direction of travel. In contrast, the savannah-living beetle *Scarabaeus lamarki* responded to the manipulation of the solar position with a corresponding change in bearing. These results suggest that the dominant compass cue used for straight-line orientation in dung beetles may be determined by the celestial cue that is most prominent in their preferred habitat.

Keywords Compass · Beetle · Orientation · Sun · Polarised light

Introduction

In most environments, the visual surroundings provide an abundance of compass cues that can be used for navigation, migration and orientation. As a distinct feature on clear days, the sun is frequently employed for this purpose (Wehner 1984; Byrne et al. 2003; Guilford and Taylor 2014; Chernetsov 2017). Similarly, the polarised skylight pattern, with the sun at its centre, also provides a reliable reference for guidance (Wehner and Muller 2006; Weir and Dickinson 2012). Light intensity and chromatic gradients, caused by intensity- and wavelength-dependent scattering of sunlight, also serve as compass cues (Rossel and Wehner 1984; Ugolini et al. 2008; el Jundi et al. 2014, 2015a). At night, the moon (Ugolini and Melis 1999), the pattern of polarised

moonlight (Dacke et al. 2003a, b, 2011), and the stars (Wiltschko et al. 1987; Mouritsen and Larsen 2001; Mauck et al. 2008; Dacke et al. 2013a; Foster et al. 2017, 2018) are used for the same purpose. In addition, many animals rely on landmarks (Collett 1996), entire skylines (Graham and Cheng 2009; Towne et al. 2017), the earth's magnetic field (Wiltschko and Wiltschko 1972; Wang et al. 2007; Dommer et al. 2008; Dreyer et al. 2018), odour (Walraff and Foa 1981; Gagliardo et al. 2013), and wind (Mueller and Wehner 2007) to find their way.

The directional information extracted from any set of compass cues can, in principle, be used in three different ways. First, an animal can use a *combination* of terrestrial and celestial cues to find its way to a goal. This can be observed in the Australian desert ants, where the ants head in an intermediate direction when terrestrial and celestial information are set in conflict (Narendra 2007; Collett 2012; Legge et al. 2014). Second, when moving over greater distances and/or over longer periods of time, different compass cues can be used in *sequence* over a length of time. A good example for this can be found in migrating birds, which follow a star compass at night, but use the sun as a compass cue when it appears in the morning sky (Muheim et al. 2003). In the same manner, on clear days, honeybees rely on celestial

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cues for directional information, but when the sky becomes overcast, these insects will rely primarily on terrestrial cues (Chittka and Geiger 1995). A third approach to compass cue use involves a 'backup system' or cue hierarchy (el Jundi et al. 2015b), where the dominant cue will be substituted by a secondary cue when no longer accessible. Pigeons, for example, use the sun as their dominant cue but rely on magnetic cues on overcast days (Walcott 2005). Similarly, most ants will primarily rely on polarised light when navigating but when experimentally deprived of this cue, they will instead orient to their secondary cue, the sun (Wehner and Muller 2006).

All diurnal, savannah-living dung beetles studied to date rely on the sun as their primary cue for orientation when transporting their dung balls away from competitors at the dung pat (Byrne et al. 2003; Dacke et al. 2013b, 2014; el Jundi et al. 2014, 2015b). As soon as the sun is out of view, which may occur due to cloud cover or vegetation, these beetles will instantaneously, with no hesitation in their stride or decrease in orientation performance, change to the next cue in the hierarchy (Byrne et al. 2003). This cue is most likely the celestial pattern of polarised light (Byrne et al. 2003; el Jundi et al. 2014). If met with a condition where neither the sun nor the polarised skylight is available, diurnal dung beetles rely on the skylight intensity gradient of the sky (el Jundi et al. 2014), and, as an apparent 'final resort', the chromatic gradient (el Jundi et al. 2015a). The compass system of nocturnal beetles follows another order, with the polarised light pattern (rather than the moon) as the primary cue (Dacke et al. 2003a; el Jundi et al. 2015b; Smolka et al. 2016). Interestingly, if coaxed into rolling their balls during the day, nocturnal beetles switch to the hierarchy of a day active beetle and orient instead to the sun as their primary cue of reference (el Jundi et al. 2015b). This suggests that the hierarchy of compass cues within the backup system of the South African beetles is dynamic, and allied to the visual ecology of the navigator.

Studies of celestial orientation in dung beetles have so far focused on South African ball rolling, savannah-living beetles that primarily orient under open, blue skies. However, ball-rolling beetles are found on all continents of the globe (except for the Antarctic), in habitats ranging from deserts to rainforests (Cambefort 1991; Scholtz et al. 2009). The visual environment of woodlands and forests differs from that of a savannah in many ways, with a core difference being the extent of overhead vegetation (Endler 1993; Shashar and Cronin 1998). The denser the canopy, the more frequently the sun will be hidden from the direct view of ground-dwelling animals. Provided that patches of clear sky are discernible, a wide-field cue, such as polarised skylight, will, however, remain equally reliable under a canopy, as under the open sky (Shashar and Cronin 1998; Hegedüs et al. 2007). Here, we examine the straight-line orientation strategy of the

woodland-living dung beetle, *Sisyphus fasciculatus*, to consider how the compass system of this species is influenced by its visual ecology.

Methods

General

With the aid of dung-baited pit-fall traps [plastic container (1 l) covered by a metal grid (30 × 30 cm)] (Tocco et al. 2017), two diurnal South African dung beetle species, *Si. fasciculatus* and *Scarabaeus (Kheper) lamarki*, were collected on Pullen nature reserve (31.10°E, 25.34°S) and Stonehenge game farm (24.32°E, 26.39°S), respectively.

To determine the vegetation type of the savannah woodland in which *Si. fasciculatus* is most abundant, three pit-fall traps were placed in the predominantly open region (dominant grass species; *Heteropogon contortus*, *Sporobolus pyramidalis* and *Chloris pycnothrix*) and three were placed in the predominantly closed region (dominant tree species; *Sclerocarya birrea*, *Searsia pentheri* and *Erythrina lysistemon*) of the beetles' habitat for three non-consecutive sample occasions during March 2018. The traps were placed along a transect 50 m apart, and baited at ground level using 400 g of fresh cow dung per trap. Beetles falling into the traps were killed by a 30% aqueous solution of ethylene glycol. Traps were emptied and re-baited with fresh dung every 6 h during daylight.

All behavioural data recorded from the field were collected in the same locations as given above, under clear skies, at solar elevations ranging between 45° and 60°, during November 2017 and March 2018. Experiments were recorded using a Sony Handycam HDR-CX730E (fitted with a 0.42 × wide-angle lens) mounted from above with the lens facing downwards. Circular statistics on measured data was performed using Oriana 3.0 (Kovach Computing Services, Anglesey, UK). All circular data are reported as mean ± circular standard deviation. All linear data are reported as mean ± standard error of the mean. The angular distribution of the change in bearings was tested using the *V* test with the expected mean of 0°, with the exception of the ersatz sun test experiment where the expected mean was determined to 180°. If the distribution of change in bearings was directed around the expected mean, the *V* test was significant.

Determining step size

Under a full view of the sky, on a flat, sand-coated, wooden surface, 20 individuals per species were allowed to roll their dung ball beside a millimetre scale. From the footage of the overhead video camera, the *x*- and *y*-coordinates of the start and end points of a stride were extracted (ImageJ1©,

National Institutes of Health, Bethesda, MD, USA). The length of a stride was determined as the distance from where the limb, controlling forward movement during ball rolling (hind leg for *Si. fasciculatus*, foreleg for *Scarabaeus lamarcki*), was steady on the arena surface, to when the same limb was seen to be steady on the surface again. True distances were obtained from the millimetre scale present in the frame. Five strides per beetle were measured to obtain an average step size for each species.

Determining orientation precision

Since the precision of orientation can be expected to weaken with an increasing number of steps (Benhamou and Bovet 1992; Cheung et al. 2007), orientation precision in the two species was measured over a radial distance corresponding to a set amount of steps (20) for the respective species (*Si. fasciculatus*: 30 cm; *S. lamarcki*: 52 cm). Under an open sky, a beetle and its ball were placed in the centre of a sand-coated, circular, wooden, arena. From here, the beetle was allowed to roll to the perimeter of the arena where its exit bearing was recorded and the beetle, with its ball, was placed back in the centre of the arena again. This was repeated 20 times for each individual and recorded from above. The mean resultant vector length (R) of these 20 exit bearings was calculated for each individual and used as a measure of orientation precision.

The paths the beetles travelled were analysed with custom-made tracking software (kindly provided by Dr. Jochen Smolka, Lund University) in Matlab R2016a (Mathworks Inc., Natick, MA, USA). A camera calibration software in Matlab was used to correct for optical distortions, and true distances travelled were obtained from a calibration pattern (3.9 × 3.9 cm, black and white squares) temporarily placed on the surface of each arena during data collection.

The hierarchy of cues in the celestial compass system

A beetle was placed alongside its dung ball on a sand-coated, circular, wooden arena, with a radius of 50 cm (*solar orientation* and *simulated solar orientation*) or 30 cm (*polarisation orientation*). The beetle was allowed to roll its ball to the perimeter of the arena, where the exit bearing was noted. This marked the end of the first trial. For the second trial, the compass cue in question (see below) was changed by 180° (*solar orientation* and *simulated solar orientation*) or 90° (*polarisation orientation*) before the beetle was placed back in the centre of the arena and allowed to roll its ball to the perimeter a second time. Here, a second exit bearing was noted. Next, a third trial, presenting the same condition as the initial trial, was performed as a control for orientation performance. To determine directional changes in the

test and control conditions, the difference in exit bearings between roll one and roll two (*test*) and roll one and three (*control*) was calculated. To avoid any influence of weather conditions, the two species were tested at the same time, alternately. A Mardia–Watson–Wheeler test was used to test for differences in the directional changes recorded for the two species. All directional statistics were obtained from Oriana 3.0 (Kovach Computing Services, Anglesey, UK).

Solar orientation

In the first trial, the beetle was placed in the centre of the arena with a full view of the sky and allowed to roll its ball to the perimeter where the bearing was recorded. For the second trial, the sun was covered from the beetle's field of view using a wooden board (100 × 75 cm) while simultaneously changing the apparent position of the sun by 180° with the aid of a mirror (30 × 30 cm).

Polarisation orientation

The beetle was placed under a circular, UV-transparent polarisation filter (BVO UV Polarizer, Bolder Vision Optik©, Boulder, CO, USA) of 30 cm radius positioned in the centre of the arena, under a full view of the sky. The filter was mounted on four legs (10 cm in height), and the edge of the filter was fitted with black cloth to prevent light entering from under the filter. The initial orientation of the filter was alternated for each beetle, with half of the beetles starting the initial trial with the polarisation filter aligned with the natural polarisation band of the sky (0°), and the other half with the filter aligned perpendicular to the natural polarisation band of the sky (90°). For the second trial, the polariser was turned by 90°.

Simulated solar orientation

In this set of experiments, the beetle was placed in the centre of a flat, wooden, circular arena, and presented with a green unpolarised light spot (LED with emission peak around 530 nm; LZ1-00G100, LedEngin, Inc., CA, USA) at an elevation of 45°, in an otherwise completely darkened room. For the second trial, the azimuthal position of the green light spot was changed by 180°.

Results

General description of *Si. fasciculatus*

Sisyphus fasciculatus (Fig. 1a) has an average body length (tip of abdomen to tip of pronotum) of 0.5 cm ± 0.01 cm, with a pronotum width of 0.3 cm ± 0.01 cm (mean ± SEM,

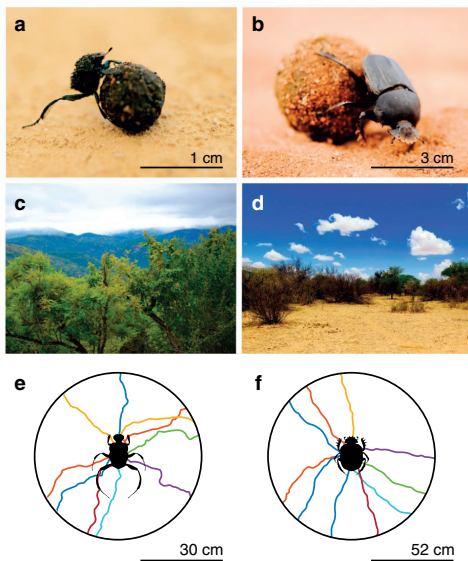


Fig. 1 Habitat and straight-line orientation in two different dung beetle species. **a** *Si. fasciculatus* and **b** *S. lamarki*, found in savannah woodland (**c**) and savannah habitat (**d**), respectively, roll their dung balls away from the dung pat on straight paths along a variety of bearings. Trajectories of ten randomly selected individuals rolling over a radial distance equivalent to 20 steps are shown for each species (**e** *Si. fasciculatus*; **f** *S. lamarki*)

$N=20$) and hind leg step size of $1.5 \text{ cm} \pm 0.1 \text{ cm}$ ($N=20$) (Fig. 1a). In comparison, its savannah-living relative *S. lamarki* has a body length of $2.86 \text{ cm} \pm 0.04 \text{ cm}$ with a fore leg step size of $2.89 \text{ cm} \pm 0.08 \text{ cm}$ (Fig. 1b). A notable difference between these species is that *Si. fasciculatus* drags its ball backwards using its hind legs, whereas *S. lamarki* pushes its ball backwards using its forelegs (Fig. 1a, b).

1175 individuals of *Si. fasciculatus* were collected within two regions of savannah woodland (Fig. 1c): open canopy area (dominated by grass) and closed canopy area (dominated by trees). In total, 821 individuals (70%) were found in the closed region, demonstrating that *Si. fasciculatus* frequently forages for dung within the closed environment of its woodland habitat.

Orientation precision of the compass system

Orientation precision in *Si. fasciculatus*

The angular direction of the first bearing of each individual when rolling 20 times across the centre of a circular arena (30 cm radius) was measured and found to be randomly

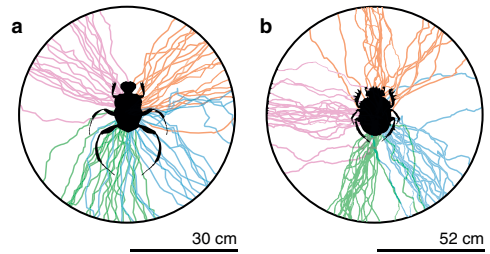


Fig. 2 Orientation performance of two different dung beetle species. Trajectories of four randomly chosen individuals rolling 20 consecutive times over a radial distance equivalent to 20 steps under a natural sky (30 cm for *Si. fasciculatus* and 52 cm for *S. lamarki*) are shown for **a** *Si. fasciculatus* and **b** *S. lamarki*. Each colour represents the trajectories of one individual from each species

distributed within the population ($P_{Si.fasciculatus} = 0.77$, Rayleigh uniformity test, $N=20$) (Fig. 1e).

Next, the ability of *Si. fasciculatus* to repeatedly orient along its chosen bearing (Fig. 2a) was investigated by calculating the mean vector length (R) obtained for each individual when rolling 20 times across the centre of a circular arena (30 cm radius), resulting in an overall mean vector length of 0.90 ± 0.02 ($N=20$) for the population (Fig. 2a).

Orientation precision in *S. lamarki*

Similar to above, the exit bearings for *S. lamarki* were randomly distributed in all directions ($P_{S.lamarcki} = 0.45$, Rayleigh uniformity test, $N=20$) (Fig. 1f). *S. lamarki* were equally as capable of maintaining a constant bearing direction over consecutive rolls as *Si. fasciculatus*, with a mean vector length for the population of $R = 0.91 \pm 0.02$ ($N=20$) (Wilcoxon rank sum test; $P=0.30$, $N=20$) (Fig. 2b).

The hierarchy of cues in the celestial compass system

The role of the sun in the celestial compass system of *Si. fasciculatus*

Next, we investigated the role of the sun in the compass system of *Si. fasciculatus*. When allowed to roll once across the arena under an unobscured sky, followed by a second time under a manipulated sky, where the sun had been mirrored by 180° and the real position of the sun was hidden from the beetle's view (*test*), no significant change in bearing was observed ($\mu_{Si.fasciculatus} = 354.1^\circ \pm 24.1^\circ$, V test (with an expected mean of 0°); $P_{Si.fasciculatus} < 0.001$, $V=0.98$, $N=30$) (Fig. 3a, top graph, yellow data points). This suggests that *Si. fasciculatus* either does not use the sun as a compass

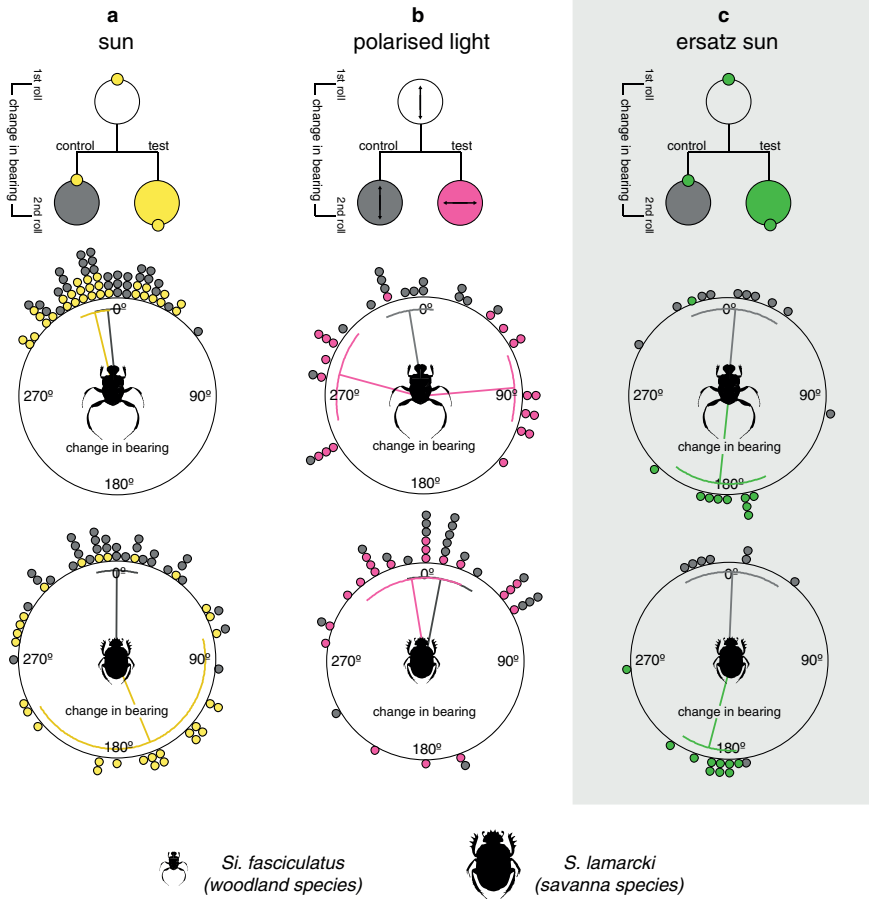


Fig. 3 Response to directional changes of compass cues. *Si. fasciculatus* and *S. lamarcki* were allowed to roll their dung balls to the perimeter of a circular arena under an open sky (**a**, **b**) or in the presence of an artificial sun in an indoor lab (**c**). When the beetle reached the periphery of the arena, the exit angle was noted and the beetle was placed back at the centre again, now with the test cue (sun, polarised light or artificial sun) moved to a different position. **a** The apparent position of the sun was changed by 180° using a mirror; **b** the apparent e-vector direction of the celestial polarisation pattern was turned 90° with a polarising filter; **c** the position of the ersatz sun (green light) was switched by 180°. The difference between the two exit angles defines the response to the treatment (**a** 180° change in sun position, yellow circles; **b** 90° change in the direction of polarisation, magenta circles; **c** 180° change in ersatz sun position, green circles). Under the mirrored sun, *S. lamarcki* responded by a change

in exit bearing approaching 180° (yellow line), while *Si. fasciculatus* showed no significant change in bearing (yellow line). When turning the e-vector by 90°, *Si. fasciculatus* showed a clear response (magenta line), while *S. lamarcki* did not respond to this treatment (magenta line). Both species responded with a change approaching 180° (green line) in response to a 180° change in position of the ersatz sun indoors. After the second exit bearing was noted, the beetle was placed back at the centre again and allowed to exit the arena a third time, now with the cue rotated back to its initial position. The angular changes between the first and third trials (*control*) are indicated by *grey circles* in all treatments. No significant change in exit bearing was observed, which indicates that beetles attempted to adhere to the same initial bearing throughout the experiment. Error bars represent one circular standard deviation

cue, or does not use the sun as its *primary* cue for orientation. The change of bearing direction was also calculated for each individual beetle between the first roll and third roll, both made under an unobscured sky (*control*). Under this condition, the average change of bearing was around 0° ($\mu_{Si.fasciculatus} = 346.2^\circ \pm 27.5^\circ$ (mean \pm circular SD), *V* test (with an expected mean of 0°); $P_{Si.fasciculatus} < 0.001$, $V = 0.87$, $N = 30$) (Fig. 3a, top graph, grey data points).

The role of the sun in the celestial compass system of *S. lamarcki*

When *S. lamarcki* was allowed to roll once under an unobscured sky, followed by a roll under the manipulated sky (in an identical setup as for *Si. fasciculatus* above), these beetles showed a marked response to the apparent 180° change in solar azimuth, with an average change in bearing of $157.5^\circ \pm 106.5^\circ$, $N = 30$ (Fig. 3a, bottom graph, yellow data points). This change in bearing is significantly different from the lack of response recorded for *Si. fasciculatus* (Mardia–Watson–Wheeler test; $P < 0.001$, $N = 30$, $W = 29.8$) (Fig. 3a). Similar to *Si. fasciculatus*, *S. lamarcki*, displayed no change in bearing direction when rolling repeatedly under an unobscured sky ($\mu_{S.lamarcki} = 0.5^\circ \pm 38.294^\circ$, *V* test (with the expected mean of 0°); $P_{S.lamarcki} < 0.001$, $V = 0.87$, $N = 30$) (Fig. 3a, bottom graph, grey data points).

The role of dorsal polarised light in the celestial compass system of *Si. fasciculatus*

In the next set of experiments, each beetle rolled twice under a polarising filter in the presence of the sun, with the filter rotated by 90° between rolls. Under these conditions, *Si. fasciculatus* changed their bearing by $82.8^\circ \pm 30.5^\circ$ ($N = 20$), suggesting this species orientates to a dorsal pattern of polarised light (Fig. 3b, top graph, magenta data points). When instead rolling two times consecutively under the polarising filter when held in place (*control*), no significant change in direction was observed ($\mu_{Si.fasciculatus} = 350.7^\circ \pm 35.5^\circ$, *V* test (with an expected mean of 0°); $P_{Si.fasciculatus} < 0.001$, $V = 0.07$, $N = 20$) (Fig. 3b, top graph, grey data points).

The role of dorsal polarised light in the celestial compass system of *S. lamarcki*

When the same test was repeated with *S. lamarcki*, these beetles only changed their direction by $45.3^\circ \pm 49.7^\circ$ (Fig. 3b, bottom graph, magenta data points), which was significantly smaller than the change in direction recorded for *Si. fasciculatus* (Mardia–Watson–Wheeler test; $p < 0.001$, $W = 14.421$, $N = 20$). Again, there was no significant change in the bearing direction between two consecutive rolls when the polarisation filter remained in the same orientation (*control*)

($\mu_{S.lamarcki} = 10.83^\circ \pm 49.3^\circ$, *V* test (with an expected mean of 0°); $P_{S.lamarcki} < 0.001$, $V = 0.68$, $N = 20$) (Fig. 3b, bottom, grey line).

Sisyphus fasciculatus can orient to an ersatz sun indoors

To investigate if *Si. fasciculatus* is able to maintain its bearing direction using a single point-light source (such as the sun) as a compass cue, individuals were presented with a green light spot as an ersatz sun (el Jundi et al. 2015a) in an indoor arena in the absence of other visual cues. When maintained in the same position over two consecutive rolls (*control*), no significant change in direction between the two rolls could be observed ($\mu_{Si.fasciculatus} = 5.1^\circ \pm 39.9^\circ$, *V* test (with the expected mean of 0°); $P_{Si.fasciculatus} < 0.001$, $V = 0.78$, $N = 10$) (Fig. 3c, top graph, grey data points), indicating that *Si. fasciculatus* is able to maintain its bearing with reference to a single point-light source if this is the only cue available. When the position of the ersatz sun was changed by 180° between two rolls, *Si. fasciculatus* changed its bearing accordingly ($185.9^\circ \pm 41.7^\circ$, $N = 10$, *V* test (with the expected mean of 180°); $P_{Si.fasciculatus} \leq 0.001$, $V = 0.77$, $N = 10$) (Fig. 3c, top graph, green data points). In contrast to the large spread in bearings recorded for *Si. fasciculatus* outside (Fig. 1e), the spread of bearings travelled indoors was significantly clustered ($P_{Si.fasciculatus} = 0.026$, Rayleigh uniformity test, $N = 20$) with a mean of $24.8^\circ \pm 65.33^\circ$ (mean \pm circular SD) relative to the azimuth of the ersatz sun. That is, the beetles could be observed to travel along bearings in the direction of the ersatz sun.

Scarabaeus lamarcki can orient to an ersatz sun indoors

When tested in the same indoor arena as above, with the ersatz sun maintained in position, also *S. lamarcki* maintained their bearings between rolls ($\mu_{S.lamarcki} = 2.4^\circ \pm 43.2^\circ$, *V* test (with the expected mean of 0°); $P_{S.lamarcki} < 0.001$, $V = 0.75$, $N = 10$) (Fig. 3c, bottom graph, grey data points). In addition, these beetles responded to a 180° change in “solar position” by a similar change in bearing as *Si. fasciculatus* ($194.9^\circ \pm 24.7^\circ$; $N = 10$) (Mardia–Watson–Wheeler test; $P = 0.31$, $W = 2.36$, $N = 10$) (Fig. 3c, bottom graph, green data points). To investigate if also the bearing directions of *S. lamarcki* were directed towards the position of the ersatz sun, the angular direction of the first bearing of each individual was measured. In this species, the bearings taken indoors were randomly distributed within the population ($P_{S.lamarcki} = 0.35$, Rayleigh uniformity test, $N = 20$), showing no significant difference from the distribution of roll bearings travelled under the natural sun (Mardia–Watson–Wheeler test; $P = 0.28$, $W = 2.52$, $N_{Greenlight} = 10$, $N_{Sun} = 20$).

Discussion

Bearing directions of *Si. fasciculatus* and *S. lamarcki*

Despite a large difference in body size and their rolling techniques [where the savannah woodland species, *Si. fasciculatus*, drags its ball backwards using its hind legs for traction (Fig. 1a), and the savannah species, *S. lamarcki*, pushes its ball backwards with its forelegs in contact with the ground (Fig. 1b)], both species move away from the centre of the arena (i.e., the dung pat) in straight lines with similar orientation precision (Figs. 1e, f, 2). The initial bearings travelled by different individuals were randomly distributed in all the directions for each species, clearly demonstrating that neither *Si. fasciculatus*, nor *S. lamarcki*, use a certain species-specific direction when orienting away from the pat, but select their bearing direction on an individual level. For *S. lamarcki*, this bearing is reset when a new ball is made, after which the beetle can be observed to roll along a different bearing (Baird et al. 2010). Whether this is also the case for *Si. fasciculatus* remains to be investigated.

Different strategies for compass cue integration

When the band of polarised light was set in conflict to the position of the sun, *Si. fasciculatus* turned in accordance to the 90° rotation of the polariser, while *S. lamarcki* showed a significantly weaker response to this manipulation. When the sun was mirrored, *Si. fasciculatus* did not respond to the positional change of this compass cue (Fig. 3a, top). This stands in contrast to past studies on the compass system of other diurnal, savannah-living, ball-rolling dung beetles that all (including the present observation of the solar compass in *S. lamarcki*) show a large change in rolling bearing in response to a manipulation of the sun's position (Byrne et al. 2003; Dacke et al. 2014; el Jundi et al. 2015b).

The savannah woodland biome, inhabited by *Si. fasciculatus* (Paschalidis 1974), differs greatly from that of the open savannah, inhabited by *S. lamarcki* (Ospina-Garcés et al. 2018), with a greater tree density and a more closed canopy in the woodland (Fig. 1c, d). In addition, *Si. fasciculatus* frequently forages within the closed region of its environment (dominant tree species; *S. birrea*, *S. pentheri*, and *E. lysistemon*, see “Methods”). The higher annual rainfall in this biome compared to the savannah (Paschalidis 1974; Rutherford et al. 2006) also suggests a higher occurrence of clouds. While overhead vegetation and clouds will hinder the use of a solar compass, the celestial polarisation pattern will remain visible under the forest canopy

(Shashar and Cronin 1998; Hegedüs et al. 2007) as well as underneath clouds, if portions of the sky can be glimpsed (Pomozi et al. 2001). Consequently, the celestial polarised light pattern is likely to be the more reliable compass cue in this type of environment.

Under the appropriate circumstances, the compass system of the woodland-living beetle is also able to obtain directional information from a point-light source. When presented with a single green light spot, a valid replacement for the real sun to a beetle (el Jundi et al. 2015a), *Si. fasciculatus* and *S. lamarcki* changed their bearings according to the azimuthal displacement of this light (Fig. 3c). The bearings chosen by *Si. fasciculatus* in response to the ersatz sun were, however, primarily directed towards the green light, while *S. lamarcki* could be observed to exit the arena along randomly distributed bearings (but see el Jundi et al. 2015a, 2016). This suggests that, under these laboratory conditions, *Si. fasciculatus* adapted a positive phototaxis rather than the menotactic behaviour observed outdoors (Fig. 1e). While more detailed investigations are required to determine if *Si. fasciculatus* can use the sun as a compass cue when orienting outdoors, we can conclude that the primary celestial cue for orientation differs between *Si. fasciculatus* and *S. lamarcki*.

In summary, this and the previous studies of the compass system in dung beetles suggest that the hierarchy of celestial cues varies with the visual ecology of the species. This appears to be true for species from different biomes (Buhlmann et al. 2011), as well as for a single species when night turns into day (el Jundi et al. 2015b). If the primary cue within each system is also the cue that supplies the compass with the highest degree of precision, it will be the focus of our next study.

Guided movement in cluttered environments

While a bare environment, such as a salt pan or a desert, can be nearly void of landmarks, the amount of tall vegetation in forests and savannah woodlands provides a large range of terrestrial cues that can be used for directional information (Hölldobler 1980; Hironaka et al. 2008; Reid et al. 2011; Rodrigues and Oliveira 2014). Not surprisingly, forest-living ants and bees rely heavily on terrestrial cues when finding their way back home (Warrant et al. 2004; Fleischmann et al. 2018a, b). In parallel, the sub-social shield bug and the African stink ant will change their bearings in response to a rotation of an artificial canopy pattern (Hölldobler 1980; Hironaka et al. 2008). Even though not directly manipulated in this study, we do not see any indication that the savannah woodland beetles stabilise their course in relation to the rich visual scenery around them. This supports earlier studies on the compass system of the dung beetles which have been shown to ignore landmarks for straight-line

orientation (Dacke et al. 2013b). An important distinction between ball-rolling dung beetles and the homing insects considered above is that, instead of repeatedly finding their way back to a well-known point in space in the form of a nest, the beetles rather set and follow a once-off course towards an unknown goal in an unfamiliar terrain to bury their ball. For such a task, landmarks have little value and provide no guidance. Instead, the compass of the woodland-living beetle *Si. fasciculatus* relies on polarised skylight as its dominant directional cue when negotiating its vegetated surroundings. Further studies of the compass system of woodland living beetles will investigate if this strategy is widely adopted by straight-line orienting insects foraging in cluttered environments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experiments in this study were performed in accordance with the ethical standards referred by the South African and Swedish guidelines for animal experiments.

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Paper III



Article

Compass Cue Integration and Its Relation to the Visual Ecology of Three Tribes of Ball-Rolling Dung Beetles

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Simple Summary: To escape competition for food at the dung pat, ball-rolling dung beetles shape a piece of dung into a sphere and roll it away. To maintain their bearing, these beetles integrate directional information from a range of celestial cues. For the majority of diurnal dung beetles living in open habitats, the most dominant of these cues is the sun. It has recently been demonstrated that beetles living in closed habitats, with closely spaced trees and tall grass, rely predominantly on directional information provided by polarised skylight rather than the sun. Taken together, these findings suggests that the orientation strategy of the beetle is influenced by the animal's visual ecology. To further investigate the relative weighting of cues in the orientation system of beetles, and its relation to their visual ecology, we investigated the orientation strategy of ball-rollers from three different dung beetle tribes, all present within the same savanna biome. We find that species within a tribe share the same orientation strategy, but that this strategy differs across tribes. We conclude that, despite dramatic intertribal differences in body size and external eye design, the dynamic heading direction network of the South African ball-rolling dung beetles is well adapted to guide the foraging insect in the habitat that it normally traverses.

Abstract: To guide their characteristic straight-line orientation away from the dung pile, ball-rolling dung beetles steer according to directional information provided by celestial cues, which, among the most relevant are the sun and polarised skylight. Most studies regarding the use of celestial cues and their influence on the orientation system of the diurnal ball-rolling beetle have been performed on beetles of the tribe Scarabaeini living in open habitats. These beetles steer primarily according to the directional information provided by the sun. In contrast, *Sisyphus fasciculatus*, a species from a different dung-beetle tribe (the Sisyphini) that lives in habitats with closely spaced trees and tall grass, relies predominantly on directional information from the celestial pattern of polarised light. To investigate the influence of visual ecology on the relative weight of these cues, we studied the orientation strategy of three different tribes of dung beetles (Scarabaeini, Sisyphini and Gymnopleurini) living within the same biome, but in different habitat types. We found that species within a tribe share the same orientation strategy, but that this strategy differs across the tribes; Scarabaeini, living in open habitats, attribute the greatest relative weight to the directional information from the sun; Sisyphini, living in closed habitats, mainly relies on directional information from polarised skylight; and Gymnopleurini, also living in open habitats, appear to weight both cues equally. We conclude that, despite exhibiting different body size, eye size and morphology, dung beetles nevertheless manage to solve the challenge of straight-line orientation by weighting visual cues that are particular to the habitat in which they are found. This system is however dynamic, allowing them to operate equally well even in the absence of the cue given the greatest relative weight by the particular species.

Keywords: orientation; orientation strategy; visual ecology; dung beetle; compass cues



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1. Introduction

To successfully navigate the world, animals often rely on directional information from more than one sensory channel [1–3]; fruit flies require visual feedback to localize an odour source [4], bees are better at pin-pointing their nest entrance with the addition of olfactory cues [5] and Bogong moths use the Earth's magnetic field in combination with landmarks to localize Alpine caves [6]. In addition, orienting insects seem to afford the greatest weight to the directional information that conveys the highest certainty at a given moment [7]. Monarch butterflies primarily rely on the sun to find their route across the North American continent [8] but will refer to polarised skylight for directional guidance as soon as this bright solar cue is obstructed [9–11], and as the sun climbs high in the sky, becoming less reliable for directional input, dung beetles and ants rely more heavily on directional information provided by wind [1,12]. Homing ants, which find their way back to their nest by path integration and landmarks, also employ a dynamic strategy for reliable navigation; as these foragers are displaced further and further from their nest and the visual scenery around them becomes increasingly unfamiliar, they shift the relative directional weight of their path integrator and landmark guidance in favour of the former to lead them back home [13].

Not surprisingly, the visual ecology of an animal influences what directional cues to follow where and when. Rodent and fish species living in spatially complex environments will rely more on egocentric cues to find their way compared to species inhabiting more open habitats [14–16]. Another example can be found among ants, where species inhabiting cluttered, landmark-rich spaces, rely more on landmark guidance compared to desert ants that forage in open, featureless habitats [13,17–20]. While these differences in directional guidance appear to be species specific, and strictly tuned to the visual environment in which the animal lives, a dynamic influence of the visual ecology of the navigator can be observed in the strictly nocturnal, savanna-living dung beetle, *Scarabaeus satyrus* (Fabricius). During a moon-lit night, this beetle orients using polarised lunar skylight in preference to the moon, but if coerced to roll during the day, the heading direction network of the beetle shifts the relative weight of these two types of celestial cues in favour of directional information provided by the sun [21].

Diurnal ball-rolling dung beetles steer their characteristic straight-line escapes from a dung pile [22–27] by directional information provided by the sun [21,23,24,27], the polarised skylight [26,28], the gradients of intensity [28] and colour that form across the daytime sky [29], as well as the prevailing winds [1]. Our understanding of how these insects roll straight over the sun-lit savanna is largely based on behavioural, morphological and neurobiological studies of beetles from the tribe Scarabaeini [1,21,23,27,28,30] (but see [24,26,31] and below). In these studies, we repeatedly found that if the directional information from the sun is set in conflict with other celestial cues, the beetles change their bearings according to the position of the sun [23,30]. These experiments clearly demonstrate that directional information from the sun is given the greatest relative weight during straight-line orientation in these large and iconic dung beetle species. In addition, it seems that, contrary to homing ants, which choose an intermediate route when directional cues are set in conflict [12,32–36], dung beetles do not average the dictates of the directional sources, but instead predominantly rely on the directional information given the greatest weight at that time. Consequently, only when the sun is hidden from view will the Scarabaeini beetles turn in response to the rotation of an overhead pattern of polarised light [28].

It was recently shown that a savanna woodland-living species of the tribe Sisyphini, *Sisyphus fasciculatus*, displays a different behaviour; when rolling under a polarising filter under a sun-lit sky outdoors, this beetle changes its bearing in accordance with the turn of the polariser [26]. This suggests that these small beetles, which traverse litter strewn terrain under closely spaced trees or through tall grass, predominantly rely on directional information from the celestial pattern of polarised light [26]. Due to this contrasting behaviour, Khaldy et al. [26] suggested that the visual ecology of the orientation system of the different species of dung beetle, just as in ants, is influenced by their distinct habitat associations. Here, we continue to explore the relative weight of directional information in

the heading direction network of three species of dung beetles from three different tribes, foraging in the closed or open habitat of the same savanna biome.

2. Materials and Methods

2.1. Selection of Dung Beetle Species

The three species of ball-rolling dung beetles initially included in this study all occur in the savanna biome [37]: *Kheper nigroaeneus* [tribe Scarabaeini], *Garreta unicolor* (tribe Gymnopleurini) and *Sisyphus fasciculatus* (tribe Sisyphini). The addition of *G. nitens* (tribe Gymnopleurini) as a fourth test species from the same biome was inspired by the unexpected finding that the heading direction network of *G. unicolor* did not attribute the greater directional weight to either the sun or the polarised light pattern (see Section 3.6). The experiments performed with this species is thus limited to defining the relative weight of directional cues in its orientation system.

2.2. Collection and Maintenance of Animals

Beetles were collected using dung-baited pit-fall traps in the Wits University, Pullen nature reserve (closed and open habitat) (31.10° E, 25.34° S) (*Kheper nigroaeneus*, *Garreta unicolor*, *Sisyphus fasciculatus*) and Bersig Eco Estate (open habitat) (27.95° E, 24.78° S) (*Garreta nitens*), South Africa. For illustrative purposes, the sampled habitats were photographed from the air (DJI Mavic 2) and from the ground (Nikon D810 fitted with an 8 mm fisheye lens) (see Figure 1). Once collected, beetles were maintained outside, in soil-filled, transparent plastic bins, and fed with fresh cow dung every second day. Beetles taken to the Department of Biology, Lund University, Sweden, were housed in large plastic bins (50 × 36 × 27 cm) in a light- and temperature-controlled room, under a 12 h light/dark cycle at a room temperature of 26 °C.

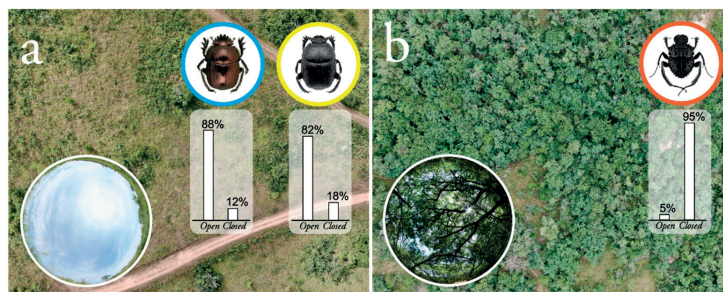


Figure 1. Dung beetles from three tribes of ball-rollers and the bioregions they inhabit. Beetles from three tribes of ball-rollers (blue-bordered image: Scarabaeini; yellow-bordered image: Gymnopleurini; red-bordered image: Sisyphini) were collected within the same savanna biome. *K. nigroaeneus* and *G. unicolor* were predominantly found actively foraging in the open habitat (a) and *S. fasciculatus* predominantly foraged within the closed habitat (b) of the same bioregion. A histogram, illustrating the percentage of individuals found in the open and closed habitat over three consecutive sampling days, is presented below each respective beetle image. A 180° view of the sky from the ground perspective of the beetle is included at the bottom of each panel.

2.3. Determining Habitat Preference and Eye Size of the Dung Beetles

2.3.1. Habitat Preference

To determine the habitat preferences of *Kheper nigroaeneus*, *Garreta unicolor* and *Sisyphus fasciculatus*, pitfall-traps were placed in the open habitat (dominant grass species: *Heteropogon contortus*, *Sporobolus pyramidalis* and *Chloris pycnothrix*) (Figure 1a) and closed habitat (dominant tree species: *Sclerocarya birrea*, *Searsia pentheri* and *Erythrina lysstemon*) (Figure 1b) for three non-consecutive sampling sessions during March 2019. Traps were

emptied and re-baited with fresh dung every 3 h during daylight hours. For more details regarding the trapping method, see Khaldy et al., 2020 [26]. The habitat preference for *G. nitens*, that shares the same savanna biome, was not defined.

2.3.2. Statistical Analysis of Habitat Preference

To test for differences in species abundance between habitat types, generalized linear mixed models (GLMMs) [38] in R (R Core Team 2020, Vienna, Austria, <https://www.R-project.org/>, accessed on 31 May 2021), used with *lme4* [39], were fitted. Each trap of each sampling event was used as a sampling unit, with a total of 155 sampling units. The Shapiro–Wilk test was used to test for normality in the residual distribution of the species abundance. The abundance of each species was non-normal count data and Poisson error distribution was specified in each model [39]. In all GLMMs, habitat type was treated as a fixed factor and sampling day as a random factor to block the layout of the sampling design.

2.3.3. Eye Size

To measure the eye surface area, the right eye of ten individuals of each species was covered with a thin layer of transparent nail polish. Once dried, the coat of nail polish was peeled off from the eye, cut and mounted flat on a microscope slide. The images of the flattened impression of the eyes were taken with a stereo microscope (Zeiss Stereo Discovery V12) and the absolute area was measured using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, MD, USA, <https://imagej.nih.gov/ij/>, 1997–2018, accessed on 20 May 2021). As *K. nigroaeneus* possesses a complete canthus, the absolute eye area for this species was calculated as the sum of the dorsal and ventral eye area.

2.4. Behavioural Experiments

Outdoors, experiments were performed under clear skies, at solar elevations between 45° and 60°, at Bersig Eco Estate and Pullen nature reserve between March 2018 and November 2019. In Lund University, Sweden, the beetles were presented with a green unpolarised light spot (Adafruit DotStar Digital LED Strip; emission peak 530 nm, Adafruit Industries, New York, NY, USA), a previously documented replacement for the sun in the heading direction network of the beetle [21], at an elevation of 45°, in an otherwise completely darkened indoor room. An overhead Sony Handycam HDR-CX730E (fitted with a 0.42× wide angle lens), mounted from above with the lens facing downwards, was used to record exit bearings.

2.4.1. Orientation Performance of Dung Beetles

To determine the beetle's orientation performance under an open sky, each individual was repeatedly placed beside its ball in the centre of a circular, flat, sand-coated arena, where the effective radius was set to a distance equivalent to the length of 20 steps for the species tested (*K. nigroaeneus*; 59 cm, *G. unicolor*; 32 cm, *S. fasciculatus*; 32 cm) (for detailed data see Supplementary Table S1). Each beetle was allowed to roll its ball to the arena perimeter ten times. Ten individuals per species were tested.

2.4.2. Relative Weighting of Directional Cues in the Orientation System of Dung Beetles

For each experimental treatment, the beetle was placed alongside its dung ball, in the centre of a 50 cm radius circular arena and allowed to roll its ball to the perimeter where its exit bearing was noted. For conditions requiring a polarising filter, a circular 30 cm radius, UV/Visible light-transparent polarisation filter (BVO UV Polarizer, Bolder Vision Optik®, Boulder, CO, USA) was positioned over the centre of the arena. The filter was mounted on four legs (10 cm in height) and fitted with a black cloth curtain around its perimeter to prevent the entry of light from outside the filter. The exit bearing was recorded when the beetle reached the filter perimeter. Upon completion of the beetle's first roll, the position of the test cue(s) was rotated by either 90° or 180° (see Section 2.4.3. below). The beetle was allowed to exit the arena and its second exit bearing was noted. A third exit, presenting the

same visual parameters as in the first trial, was performed as a control to test whether the beetle could follow approximately the same bearing throughout the experiment. Angular change was calculated as the difference in bearing between the first and second exit (*test*), or first and third exit (*control*). In total, each individual rolled from the centre to the edge of the arena (or filter perimeter) three times. In all outdoor experiments, 20 individuals per species were tested. For the indoor experiments, 10 individuals were tested for each species.

2.4.3. Manipulation of Directional Input

Sun (ersatz or real): In the field, the sun's apparent position was changed by 180° using a mirror (30 × 30 cm), while simultaneously concealing the real sun from the beetle's field of view using a wooden shade board (100 × 75 cm). Indoors, the azimuth of the ersatz sun was changed by 180° between trials by switching off and on the green light spot at different relative positions.

Polarised light: In the field, the UV/Visible light-transparent polarisation filter was turned by 90°, between consecutive rolls, either under a full view of the sun or with the sun shielded from the beetle's field of view by the shade board. The initial orientation of the filter was alternated for each beetle, with every second beetle starting with the polarisation filter aligned to the natural polarisation band of the sky, and every other beetle with the filter aligned perpendicular to the natural polarisation band of the sky.

Sun and polarised light: In these experiments, the polarising filter was turned by 90° in combination with a 180° change in the solar position, as described above.

2.4.4. Circular Statistics

Circular statistics on measured data was performed using Oriana 4.0 (Kovach Computing Services, Anglesey, UK). All circular data are reported as mean ± one circular standard deviation. Distributions of exit angles were analysed using Rayleigh's uniformity test for circular data [40]. Changes in direction between treatments were calculated by measuring the angular difference in exit bearing between two exits from the arena and analysed using a v-test with an expected mean of 0° for the control experiments and 180° for the mirrored sun/ersatz sun experiments. To test for homogeneity of two or more samples, a Mardia–Watson–Wheeler test was used.

3. Results

3.1. Habitat Preference

Kheper nigroaeneus and *G. unicolor* were primarily found actively foraging within the open habitat (open vs. closed habitat: *K. nigroaeneus*; $p < 0.001$, z-value = 8.60, estimate = 2.09, N = 165; *G. unicolor*; $p < 0.001$, z-value = 18.41, estimate = 1.55, N = 971, GLMM test) (Figure 1a, histogram), while *S. fasciculatus* was mainly found in the closed habitat (closed vs. open habitat: *S. fasciculatus*; $p < 0.001$, z-value = −19.39, estimate = −2.87, N = 939, GLMM test) of the same bioregion (Figure 1b, histogram). These findings strongly suggest that *K. nigroaeneus* and *G. unicolor* preferentially forage for dung in the open habitat, while *S. fasciculatus* forages for dung in the closed habitat of the same bioregion.

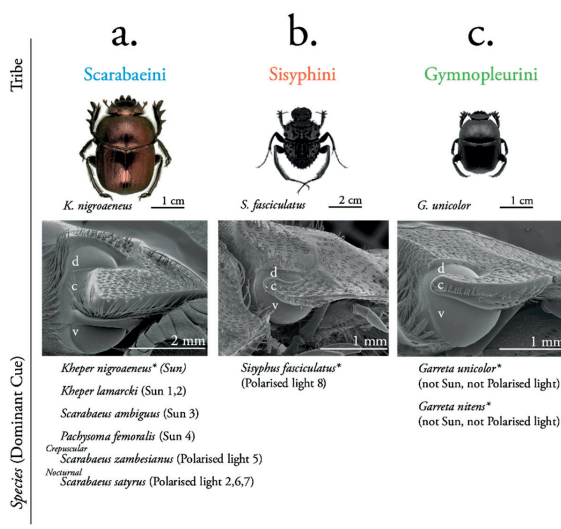
3.2. Differences in Eye Size and Shape

The relatively big eye ($1.60 \pm 0.57 \text{ mm}^2$) of *K. nigroaeneus* is completely divided into a dorsal ($0.59 \pm 0.22 \text{ mm}^2$) and a ventral part (Figure 2a), while the smaller eyes of *G. unicolor* ($0.21 \pm 0.03 \text{ mm}^2$) and *S. fasciculatus* ($0.15 \pm 0.03 \text{ mm}^2$) rather have a more oval-shaped dorsal eye (0.05 ± 0.01 and $0.02 \pm 0.01 \text{ mm}^2$, respectively) which connects to the ventral part of the eye (N = 10) (Figure 2b,c) (for detailed data see Supplementary Table S1).

3.3. Orientation Performance under the Natural Sky Is Equal for All Species

The outdoor orientation performance of the three species, as determined from the mean resultant vector length (R) of 10 exit bearings per beetle from the centre of the circular arena (the closer to 1, the better oriented the beetle) did not differ between the species

(*K. nigroaeneus*: $R = 0.93 \pm 0.1$; *G. unicolor*: $R = 0.93 \pm 0.1$; *S. fasciculatus*: $R = 0.88 \pm 0.1$, $p = 0.15$, Kruskal–Wallis test, $N = 10$) (for detailed data see Supplementary Table S2). We also found that within a species, the first bearing chosen by each individual was not biased towards any particular heading (*K. nigroaeneus*: $p = 0.06$, $Z = 2.8$; *G. unicolor*: $p = 0.54$, $Z = 0.64$; *S. fasciculatus*: $p = 0.22$, $Z = 1.53$, Rayleigh uniformity test, $N = 10$). Although the evidence for this was weaker in *K. nigroaeneus* ($p = 0.06$), previous work on closely related Scarabaeini species [22,30] suggests that this is most likely an effect of the small sample size. Taken together, this indicates that, under an open sky, our test species, from three different tribes, are able to travel along any given bearing with the same angular precision.



1. Dacke et al. 2014, *Phil Trans R Soc B*; 2. el Jundi et al. 2015, *Proc Nat Acad Sci*; 3. Khaldy et al. 2019, *Roy Soc Int*;
4. Byrne et al. 2005, *J Comp Physiol A*; 5. Dacke et al. 2005, *J Exp Biol*; 6. Dacke et al. 2011, *Phil Trans R Soc B*;
7. Fozzer et al. 2018, *J Exp Biol*; 8. Khaldy et al. 2019, *J Comp Physiol A*.

Figure 2. Habitus, eye design and dominant orientation cue in three tribes of ball-rolling dung beetles. Diurnal species within the tribe Scarabaeini (a) attribute greatest relative weight to directional information provided by the sun during straight-line orientation. In contrast, two nocturnal species from the same tribe, as well as the smaller, diurnal *Sisyphus fasciculatus*, from the tribe Sisyphini (b), rely predominantly on polarised skylight for directional information. The underlying weighting strategy for straight-line orientation within the tribe Gymnopleurini (c) differs from that previously mentioned, where neither directional information from the sun nor the polarisation pattern dominates the output from its compass network. As can also be noted from our test species, Sisyphini are generally much smaller than Gymnopleurini, which in turn are smaller than Scarabaeini. The relative eye sizes across the three tribes follow the same pattern, but they differ in shape. The canthus (c) completely separates the roughly equal sized dorsal (d) and ventral (v) eyes of the Scarabaeini, while the dorsal portion of the eye of the Gymnopleurini and Sisyphini is only partially separated and much smaller than the ventral part. Species tested in this study are indicated by an asterisk (*).

3.4. Ball-Rolling Dung Beetles Can Orient to a Single Green Light Spot

Beetles presented with a green light spot (indoors) as an ersatz sun in the same azimuthal position between two consecutive exits from the centre of the arena (*control*), and showed no significant change in direction in any of the three species, (*K. nigroaeneus*: $\mu = 28.37^\circ \pm 49.27^\circ$, $p < 0.01$, $V = 2.72$; *G. unicolor*: $\mu = 333.7^\circ \pm 54.7^\circ$, $p < 0.01$, $V = 2.54$; *S. fasciculatus*: $\mu = 5.13^\circ \pm 39.89^\circ$, $p < 0.001$, $V = 3.50$, mean \pm circular s.d., v-test (with the expected mean of 0°), $N = 10$) (Figure 3a, grey dotted vector).

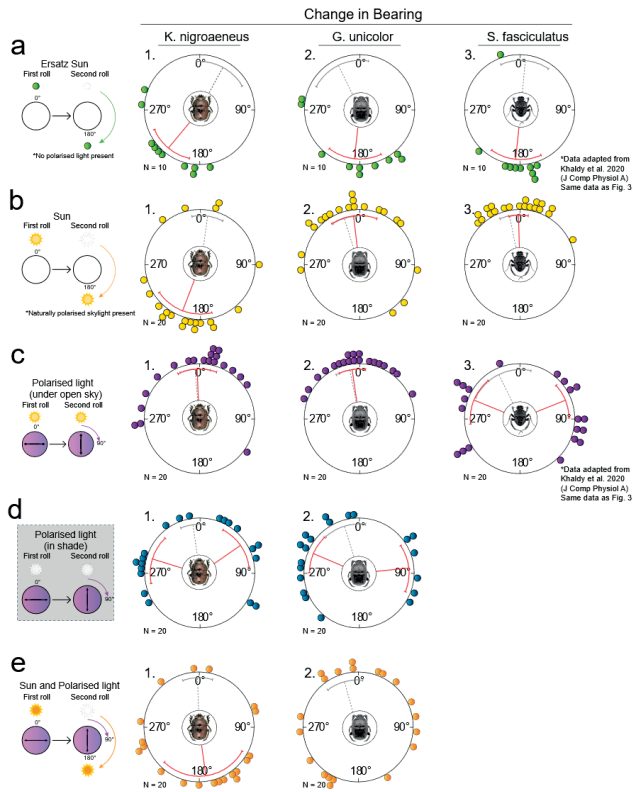


Figure 3. Response to directional change of compass cues. Three diurnal dung beetle species (from left to right; *Kheper nigroaeneus*, *Garreta unicolor* and *Sisyphus fasciculatus*) were allowed to roll balls out of a circular arena in a darkened room (a) or outdoors under the open sky (b), or with a polarisation filter placed above the arena with the sun visible (c,e) or with a polarisation filter placed above the arena with the sun shielded from view (d). Once the beetle had reached the periphery of the arena, it was removed from its dung ball and placed back in the centre alongside its ball. At this time, the apparent e-vector direction was turned by 90° using a polarisation filter ((c,d), purple arrow) or the position of the sun was changed by 180° while simultaneously turning the apparent e-vector direction by 90° ((e), orange arrow: sun; purple arrow: polarisation filter). The beetle was then allowed to exit the arena a second time. The absolute angular difference between the first and the second exit angle represent the response to the treatment (test). (a): With the ersatz sun switched by 180°, all three species changed their bearings in accordance with this angular change (red vector, all graphs); (b): with the sun mirrored by 180° outdoors, only *K. nigroaeneus* showed a significant change in bearings in response to this manipulation; (c): with the e-vector turned by 90° under a clear sky, only *S. fasciculatus* responded significantly by a change in exit bearings approaching 90°; (d): with the sun shielded from view, a significant change in bearings could also be elicited in *K. nigroaeneus* and *G. unicolor*; (e): with the sun mirrored by 180° and the polarisation pattern e-vector turned by 90°, *K. nigroaeneus* changed its exit bearings in a similar fashion to when only the sun was mirrored by 180° (see graph 1 in (b)), while the changes in exit bearings for *G. unicolor* were randomly distributed within the population (graph 2). Beetles were then allowed to roll a third time, with the manipulated cue(s) moved back to its/their initial position. The mean angular difference between the first and second exit (test), and the first and third exit (control), is represented by a red solid vector and a grey dotted vector, respectively, in each graph. Error bars represent one circular standard deviation. The data presented for *S. fasciculatus* in (a) and (c) (graph 3, respectively) were adapted from Khaldy et al., 2020 [26]. For detailed data see Supplementary Table S3.

When the position of the ersatz sun was changed by 180° between two exits from the centre of the arena (*test*), all species changed their headings accordingly (*K. nigroaeneus*: $\mu = 219.59^\circ \pm 37.73^\circ$, $p < 0.01$, $V = 2.77$; *G. unicolor*: $\mu = 186.27^\circ \pm 49.40^\circ$, $p < 0.001$, $V = 3.47$; *S. fasciculatus*: $\mu = 185.9^\circ \pm 41.67^\circ$, $p < 0.001$, $V = 3.41$, v-test (with the expected mean of 180°), $N = 10$) (Figure 3a). These changes in headings showed that the species tested can steer with reference to a single point-light source and with no significant difference in performance between species ($p = 0.139$, $W = 6.95$, Mardia–Watson–Wheeler test, $N = 10$).

3.5. The Role of the Sun in the Orientation System of Ball-Rolling Dung Beetles

When allowed to exit the arena under the open sky, followed by an exit where the apparent solar position was mirrored by 180° (*test*), *K. nigroaeneus* still showed a marked change in heading ($\mu = 201.05^\circ \pm 69.46^\circ$, $p < 0.01$, $V = 2.83$, v-test (with an expected mean of 180°), $N = 20$) (Figure 3b, graph 1). In contrast, the differences in headings travelled by *G. unicolor* and *S. fasciculatus* in response to this treatment clustered around 0° (*G. unicolor*: $\mu = 353.4^\circ \pm 59.31^\circ$, $p < 0.001$, $V = 3.68$; *S. fasciculatus*: $\mu = 358.37^\circ \pm 25.58^\circ$, $p < 0.001$, $V = 5.72$, v-test (with an expected mean of 0°), $N = 20$) (Figure 3b, graph 2, 3).

As for the control for the experimental treatment (including our handling of the beetles), the changes in bearing between two exits under an unmanipulated sky was also calculated (*control*); the average change of bearings was clustered around 0° for all species (*K. nigroaeneus*: $\mu = 8.01^\circ \pm 42.34^\circ$, $p < 0.001$, $V = 4.77$; *G. unicolor*: $\mu = 342.76^\circ \pm 32.52^\circ$, $p < 0.001$, $V = 5.14$; *S. fasciculatus*: $\mu = 348.65^\circ \pm 26.71^\circ$, $p < 0.001$, $V = 5.57$, v-test (with an expected mean of 0°), $N = 20$) (Figure 3b, grey dotted vector). In addition, no significant difference in orientation performance was observed between the test and control conditions for *G. unicolor* and *S. fasciculatus* (*G. unicolor*: $p = 0.61$, $W = 0.98$; *S. fasciculatus*: $p = 0.50$, $W = 1.4$, Mardia–Watson–Wheeler test, $N = 20$). Together, these results indicate that directional information from the sun is given a greater relative weight in the orientation system of *K. nigroaeneus* compared to that of *G. unicolor* and *S. fasciculatus*.

3.6. The Role of Polarised Light in the Orientation System of Ball-Rolling Dung Beetles

When a polarising filter was placed above the arena aligned to the dominant e-vector direction in the open sky, then followed by a 90° rotation of the filter for the second exit (or vice versa), *S. fasciculatus* changed their heading by $82.75^\circ \pm 30.50^\circ$ ($N = 20$) (Figure 3c, graph 3). In contrast, the change in headings recorded for *K. nigroaeneus* and *G. unicolor* clustered closer to 0° (*K. nigroaeneus*: $\mu = 357.64^\circ \pm 51.71^\circ$, $p < 0.001$, $V = 4.22$; *G. unicolor*: $\mu = 351.83^\circ \pm 36.02^\circ$, $p < 0.001$, $V = 5.14$, v-test (with an expected mean of 0°), $N = 20$) (Figure 3c, graph 1,2), indicating that these beetles did not respond to the 90° rotation of the polariser. This suggests that directional information from the overhead pattern of polarised skylight is given a greater relative weight in the orientation system of *S. fasciculatus* compared to that of *K. nigroaeneus* and *G. unicolor*.

When exiting twice from under a polarising filter kept in the same orientation (*control*), no significant change in direction was observed for any of the three species (*K. nigroaeneus*: $\mu = 355.16^\circ \pm 40.91^\circ$, $p < 0.001$, $V = 4.88$; *G. unicolor*: $\mu = 349.11^\circ \pm 38.87^\circ$, $p < 0.001$, $V = 4.93$; *S. fasciculatus*: $\mu = 333.71^\circ \pm 73.49^\circ$, $p < 0.01$, $V = 4.84$, v-test (with an expected mean of 0°), $N = 20$). In addition, no significant difference in orientation performance was observed when exiting twice under a unmanipulated polarising filter compared to exiting twice under an unmanipulated sky (*K. nigroaeneus*: $p = 0.84$, $W = 0.36$; *G. unicolor*: $p = 0.93$, $W = 0.15$; *S. fasciculatus*: $p = 0.99$, $W = 0.015$, Mardia–Watson–Wheeler test, $N = 20$), demonstrating that the addition of the polarisation filter did not have an effect on orientation performance (Figure 3c, grey dotted vector).

To further investigate the role of polarised skylight on the orientation system of *K. nigroaeneus* and *G. unicolor*, the polarising filter was again placed above the arena, but now with the sun obstructed from view. The changes in headings recorded for the two species in response to a 90° rotation of the filter now clustered around $67.36^\circ \pm 35.45^\circ$ for *K. nigroaeneus*, and around $72.49^\circ \pm 36.57^\circ$ for *G. unicolor* ($N = 20$) (Figure 3d), demonstrating

that when the sun is obstructed from view, directional information from the overhead polarised light pattern is now attributed a relatively greater weight in the orientation system of these two species.

3.7. The Combined Role of Sun and Polarised Skylight in the Orientation System of *Garreta unicolor* and *G. nitens*

Given that *G. unicolor* did not turn despite a displacement of the sun or rotation of the pattern of polarised light under the open sky but did orient to an ersatz sun indoors and to a polarised light pattern in the shade, we then rotated the polariser by 90° while simultaneously mirroring the sun by 180° and shielding the real sun from the beetle's view (Figure 3e, graph 2). To our surprise, the angular changes in bearing recorded for *G. unicolor* in response to this manipulation were not different from a random distribution ($p = 0.70$, $Z = 0.37$, Rayleigh uniformity test, $N = 20$) (Figure 3e, graph 2). It is important to note that the beetles still maintained a straight trajectory when rolling. As soon as the real sun was revealed and the polarising filter was turned back to its original position, the beetles resumed their initial direction of travel ($\mu = 344.67^\circ \pm 49.03^\circ$, $p < 0.001$, $V = 4.23$, v -test (with an expected mean of 0°), $N = 20$).

To further evaluate this somewhat surprising observation, we repeated this experiment on *K. nigroaeneus* and the close relative *G. nitens* (due to their experimentally frailer nature, the tiny *S. fasciculatus* would not perform under this condition, but rather flew away from the setup at any given chance). While *K. nigroaeneus* altered its heading towards a 180° turn ($\mu = 171.92^\circ \pm 85.32^\circ$, $p = 0.02$, $V = 2.07$, v -test (with an expected mean of 180°), $N = 20$) (Figure 3e, graph 1), the experimental outcome for *G. nitens* was similar to that of its congeneric: no change in bearing when the solar position was mirrored by 180° ($\mu = 358.37^\circ \pm 25.56^\circ$) or in response to the 90° turn of the e-vector ($\mu = 4.78^\circ \pm 49.91^\circ$), but a significant change in bearing when the two cues were rotated together ($p < 0.001$, $W = 18.28$, Mardia–Watson–Wheeler test, $N = 20$). Similar to our findings for *G. unicolor*, the change in bearing recorded for *G. nitens* in response to simultaneous manipulation, were randomly distributed within the population ($p = 0.44$, $Z = 0.83$, Rayleigh uniformity test, $N = 20$). The beetles returned to their initial direction of travel as soon as the cues were rotated back to their original positions ($\mu = 8.57^\circ \pm 39.90^\circ$, $p < 0.001$, $V = 4.91$, v -test (with an expected mean of 0°), $N = 20$).

4. Discussion

In this study, we demonstrated how the orientation system of ball-rolling dung beetles, belonging to three different tribes that co-occur within the same savanna biome, attribute different relative weights to directional information during straight-line orientation.

4.1. Diurnal Scarabaeini Attribute the Greatest Relative Weight to the Directional Information Provided by the Sun

As with sandhoppers, monarch butterflies and birds [41–43], ball-rolling dung beetles can direct their straight-line movements according to directional input from a single source of light in an indoor setting (Figure 3a and [21,24,27]). Outdoors, however, the beetles are exposed to a range of celestial directional cues, including the sun, polarised skylight [44–46], as well as the gradients of intensity [47] and colour [48–50] that form across the natural sky. Therefore, if the apparent position of the real sun is changed by 180° with the aid of a mirror and a shading board, the directional information from the sun is set in conflict with that of the rest of the sky. Nevertheless, *Kheper nigroaeneus* changed its roll bearing in accordance with such an experimental displacement of the sun (Figure 3b, graph 1). A comparable response to this manipulation has also been documented for three other members of the Scarabaeini; *K. lamarki* [21,23], *Scarabaeus ambiguus* (Boheman) [30] and *Pachysoma femoralis* Kirby [24], suggesting that the orientation system of these species attributes the greatest relative weight to the directional information provided by the sun.

Consistent with this observation, *K. nigroaeneus* did not respond to a 90° turn of an artificial, highly polarised pattern of polarised light, when presented from above in full view

of the unmanipulated sun (Figure 3c, graph 1). However, as soon the sun was hidden from view, the beetles showed a clear 90° turn in response to the rotated polariser (Figure 3d, graph 1). It appears that once the sun is absent, which also naturally happens when it is obscured by a passing cloud, the distribution of the relative weight between the directional cues that remained can shift in favour of the polarised light input (Figure 3d, graph 1). The same holds true also for the close relative, *K. lamarcki* [28], where a behavioural response to the directional input from the gradients of colour and intensity can be seen when presented in isolation [28,29].

4.2. *Sisyphus fasciculatus* Attributes Greatest Relative Weight to the Directional Information Provided by the Celestial Polarisation Pattern

Neither *Garreta unicolor* nor *S. fasciculatus* changed their bearings according to the displacement of the sun (Figure 3b, graph 2, 3), indicating that the relative weight attributed to this directional cue in their orientation system is somewhat lower. This supports the results of a recent study [26], where, in contrast to *Kheper nigroaeneus*, *S. fasciculatus* turns in accordance with the turn of the polariser under a natural sky. Together, these findings clearly demonstrate that the smaller *S. fasciculatus* attributes the greatest relative weight to the directional information provided by the (artificial) linear pattern of polarised light. This sky-wide celestial cue is also known to play a significant role in the orientation system of other insects (locusts [51], honeybees [52] and bull ants [32]), and in some cases, even plays a dominant role (nocturnal dung beetles [21,53,54], flies [55] and desert ants [56]).

4.3. A Different Weighting of Directional Reference Cues in *Garreta* Species

To our surprise, *G. unicolor* kept to its original direction of travel both in the presence of a mirrored sun (Figure 3b, graph 2), and under a turned polariser (Figure 3c, graph 2). Only when these two cues were rotated in combination did this species demonstrate a behavioural response, which was an angular change in bearing which appeared to be randomly distributed within the population (Figure 3e, graph 2). A similar response could be confirmed in its congeneric, *G. nitens* (), suggesting that this is a tribe-specific orientation strategy.

Due to experimental constraints, this combined manipulation of directional information from the sun and the over-head pattern of polarisation was achieved by a 180° shift in the apparent position of the sun in combination with a 90° rotation of the polarisation pattern. The outcome of these manipulations was that the position of the two cues were not only changed in relation to the unmanipulated gradients of intensity and colour that spans the sky, but also in relation to each other. This drastic and multi-angular change in directional input could potentially cause the beetles to simply re-set their roll bearings, effectively contributing to the random changes in bearings displayed by *G. unicolor* (Figure 3e). However, this conjecture can be refuted, as the beetles faithfully returned to their initial bearings as soon as the cues were returned to their initial positions (Figure 3e, grey dotted vector, graph 2). Additionally, when tested under the same multi-conflict paradigm, *K. nigroaeneus* showed a clear and directed response. Attributing the greatest relative weight to the sun, these beetles simply continued to follow the angular displacement of this cue also under this experimental condition (compare Figure 3b,e). One possibility is of course that in our experiments with *G. unicolor*, each beetle followed an individual strategy; some turned 180° according to the sun, some 90° according to the overhead polarisation and some followed the stable gradients of intensity and colour. This is, however, unlikely, as we would then have expected to see a different and much more varied response when these cues were manipulated on their own (see Figure 3b,c). The random spread of changes in bearings observed for the *Garreta* sp. rather points to a more even weighting of directional information where the combined directional information in this artificial cue conflict experiment, results in a weak directional signal. While the beetles were still able to exit from the centre of the arena along straight paths, small, individual differences in the weighting of cues could now be seen in large differences in angular change. It would have been interesting to evaluate this theory further by testing the same beetle repeatedly before

rotating all cues back to their initial positions again, but this was unfortunately not within the scope of this study.

While our results do not reveal the precise nature of the orientation strategy of the *Garreta* species, we can still conclude that the heading direction networks of our three test species process the directional information provided by the sky somewhat differently; *K. nigroaeneus* preferentially steers according to the sun, *S. fasciculatus* with the pattern of polarised light, and *G. unicolor* (and *G. nitens*) does not attribute a greater relative weight to either of these cues.

4.4. Compass Cue Integration and Its Relation to the Visual Ecology of Ball-Rolling Dung Beetles

Given that a navigator can reliably perceive and analyse directional information provided by the sun and its pattern of polarised light, neither of these cues should be inherently more reliable for orientation than the other. We previously showed that ball-rolling beetles that attribute the greatest weight to directional information provided by the sun, are equally well directed in its absence when an alternative cue is available [23,28]. This holds true also for ants [56], monarch butterflies [9] and fruit flies [55]. It is further important to note, that the three tribes of dung beetles tested in this study—each attributing a different relative weight to the sun and the celestial polarised light pattern—all orient with the same precision under a clear, open sky (Figure 2b, grey mean vector (*control*)). Taken together, this indicates that the directional information provided by the sun or polarised light in the photon-rich African sky can (i) support orientation with the same precision, and (ii) be processed with comparable accuracy by the visual system and heading direction network of the Scarabaeini beetles. This is most likely also the case for the Gymnopleurini (species *G. unicolor* and *G. nitens*), that do not seem to employ differential weighting to any of the celestial cues tested.

Even though our test species are active within the same bioregion, *K. nigroaeneus* and *G. unicolor* were found actively foraging in the open habitat (Figure 1a), while the smaller *S. fasciculatus* rather foraged for dung within the closed habitat (Figure 1b). In this habitat, with tall grass and a high density of trees, the sun will be frequently obstructed from view, while a wide-field cue, such as the celestial polarised light pattern, will remain visible through any overhead vegetation [45,57,58]. This is also the cue attributed the highest directional weight in the orientation system of *S. fasciculatus* [26]. While it would have been preferable to explore the orientation strategy of additional species within the tribe Sisyphini from a different visual habitat, this unfortunately proved impossible as the species available to us (*Sisyphus manni* [59] and *Sisyphus seminulum* [60]) are so small (pronotum width: 3–5 mm) and timid, that not even the most experienced beetle experimentalist could coerce them into performing in our experiments. Still, our limited results from this tribe again suggest that they afford the greatest weight to the most consistent source of celestial directional information in their cluttered habitat [7], a strategy also found in ants [18,19].

As day turns into night, the visual world changes drastically, most notably in the decrease in light intensity [61]. At this time of the day, visually driven orientation systems need to capture as much light as possible. One common way to meet this challenge is by an increase in eye size [62,63], but it is also interesting to note that some neurons within the heading direction network of the desert locust have a higher absolute sensitivity to polarised than to unpolarised light [64]. The larger eyes of the nocturnal, open habitat Scarabaeini beetles (*Scarabaeus satyrus* and *S. zambesianus*), possess a large dorsal rim area (DRA) (the polarisation sensitive region known to detect polarised light in insects [65–67]) and rely on directional information from the polarised skylight above that provided by the moon itself [25,53,54]. In contrast, the diurnal *K. lamarcki*, which is active in the same habitat, only possess a single dorsal row of polarisation-sensitive ommatidia (Dacke *unpublished data*). The orientation systems of beetles active under more challenging light conditions—in the dark or under vegetation canopies—thus seem well adapted to their respective visual ecologies.

It is interesting to note that differences in external eye morphology between the ball-rolling beetles are more pronounced between the three tribes, than within the tribes

themselves [68] (Figure 2). As representatives of their respective tribes ([69–71]), *S. fasciculatus* and *G. unicolor* possess a more oval-shaped dorsal eye compared to that of *K. nigroaeneus*, where the dorsal eye of the medium-sized *G. unicolor* is proportionally smaller than that of *K. nigroaeneus*, while the small and spindly *S. fasciculatus* has the smallest dorsal eye of the three (Figure 2b). If we would assume that the small and narrow dorsal eyes of the smaller species also have a smaller visual field [72–76], the heading direction networks of the narrow-eyed Sisyphini and Gymnopleurini could possibly benefit from a sky-wide orientation signal, such as the celestial polarisation pattern, rather than using the position of a single light source. These inter-tribal differences might be an additional influence on how species within each tribe weight the sources of directional information they can reliably use.

In conclusion, despite exhibiting different body size, eye size and morphology, dung beetles nevertheless manage to solve the challenge of straight-line orientation by weighting visual cues that are particular to the habitat in which they are found. This system is however dynamic, allowing them to operate equally well even in the absence of the cue given the greatest relative weight by the particular species.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/insects12060526/s1>, Table S1: Eye area and step size for *K. nigroaeneus*, *G. unicolor* and *S. fasciculatus*; Table S2: Exit angles (0–359°) of ten exit rolls of ten individuals of *K. nigroaeneus*, *G. unicolor* and *S. fasciculatus*, respectively, and the corresponding calculated mean vector length (R); Table S3: Absolute angular difference in exit bearing of each manipulation (*test*: first and second exit bearing; *control*: first and third exit bearing) for each individual beetle, in each paradigm; Supplementary Figure S1: Response to the directional change of compass cues in *G. nitens*. (<https://doi.org/10.5281/zenodo.4891096>, accessed on 1 June 2021).

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Paper IV



The interplay of directional information provided by unpolarised and polarised light in the heading direction network of *Kheper lamarcki*

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Abstract

The sun is the most prominent source of directional information in the heading direction network of the diurnal, ball-rolling dung beetle *Kheper lamarcki*. If this celestial body is occluded from the beetle's field of view, the distribution of the relative weight between the directional cues that remain shifts in favour of the celestial pattern of polarised light. In this study, we continue to explore the interplay of the sun and polarisation pattern as directional cues in the heading direction network of *K. lamarcki*. By systematically altering the intensity and degree of the presented polarised light cue, we effectively change the relative reliability of these two directional cues as they appear to the dung beetle. The response of the ball-rolling beetle to these modifications allows us to closely examine how the weighting relationship of these two sources of directional information is influenced and altered in the heading direction network of the beetle. We conclude that the process in which *K. lamarcki* relies on directional information is very likely done based on Bayesian reasoning, where directional information conveying the highest certainty at a particular moment is afforded the greatest weight.

Introduction

Combining information from several different sensory cues can reduce the effect of noise in a system, allowing for greater accuracy of the behavioural output (Cheng et al., 2007; Deneve and Pouget, 2004). Within the realm of navigation, multisensory integration provides a robust navigational toolkit that lowers directional uncertainty; rock ants follow less tortuous routes when landmarks are visible (Hunt et al., 2018) and desert ants are better at localizing their nest when olfactory cues are present (Huber and Knaden, 2017). Depending on the context and conditions under which the animal finds its way, directional information from multiple sensory cues can often be integrated, operating in parallel (Buehlmann et al., 2020). Thus, navigational performance will not be compromised if directional information from one source is disrupted. At high solar elevations, when directional information from the sun is deemed unreliable (Dacke et al., 2014), dung beetles rely on directional information from wind to guide their straight-line orientation across the savanna (Dacke et al., 2019). Similarly, *Myrmica* ants, that predominantly depend on directional information from visual cues when negotiating a maze, resort to olfactory cues for directional information as the light intensity decreases and visual information becomes less reliable (Cammaerts, 2012).

The process in which orienting and navigating insects integrate multiple sources of directional information is very likely done according to Bayes' theorem (Körding, 2007; Körding and Wolpert, 2006): directional information conveying the highest certainty at any given moment is afforded the greatest weight in the navigational network of the animal. In homing ants, which find their way back to their nest by path integration (PI) and landmark guidance (LG), the weighting relationship of the PI and LG will shift in favour of the former as the ants are displaced further from their nest (Wystrach et al., 2015). With growing distance, the surrounding visual scenery becomes increasingly unfamiliar, while at the same time the ant's PI vector becomes longer, providing a stronger, more reliable source of information. Along the same line of reasoning, if two directional cues of equal weight are set in conflict, this should result in an intermediate direction between both sources of information. This outcome is observed in homing ants when the apparent e-vector direction of the celestial pattern of polarised light is set in conflict with the artificial panorama (Freas et al., 2017; Reid et al., 2011) or the artificial panorama is set in conflict with celestial cues (Legge et al., 2014; Wystrach et al., 2015).

For the dung beetle *Kheper lamarcki*, the sun is naturally the most prominent directional compass cue in its heading direction network (Dacke et al., 2013a; Dacke et al., 2014; el-Jundi et al., 2015; Khaldy et al., 2019a; Khaldy et al., 2019b; Smolka et al., 2016). If the position of the sun is experimentally set in conflict with other celestial cues (with the aid of a mirror), *K. lamarcki* changes its bearing by 180° in response to this positional change (Dacke et al., 2014). Comparably, if the view of

the sun is blocked (by a shading board), and the e-vector direction of the celestial polarised light is turned by 90° with a polariser, this beetle turns in accordance with the 90° positional change of the e-vector. Thus, when the sun is out of sight, the relative weight between the remaining directional cues shifts in favour of the celestial pattern of polarised light (el-Jundi et al., 2014).

In this study, we explore in greater detail, the interplay of the sun and polarisation pattern as directional cues in the heading direction network of the beetle. Following the rather unusual finding reported in Dacke et al., 2002, where UV and green receptors were found in the dorsal rim area (DRA) of a closely related diurnal dung beetle *Pachysoma striatum*, we also set out to measure the spectral sensitivity of the DRA of *K. lamarcki*. By altering the intensity and degree of the presented polarised light cue, we effectively change the reliability of the cues as they appear to the dung beetle, allowing us to examine how the weighting relationship of these two sources is influenced and altered by their reliability in the heading direction network of the beetle.

Material and Methods

Collection and Maintenance of Animals

Beetles of the diurnal species *Kheper lamarcki* were collected using dung-baited pit-fall traps at Stonehenge game farm (26°23'56"S, 24°19'36"S), South Africa, in November 2020 and February 2021. Once collected, beetles were transported to the Department of Biology, Lund University, Sweden, and housed in large plastic bins (50x36x27 cm) in a light- and temperature-controlled room, under a 12 h light/dark cycle, at a room temperature of 26°C and fed with fresh dung every third day.

Statistics

Circular data are reported as mean \pm one circular standard deviation. Circular statistics on measured data were performed using Oriana 4.0 (Kovach Computing Services, Anglesey, UK). Distributions of exit angles were analysed using Rayleigh's uniformity test for circular data (Batschelet, 1981). Changes in direction between treatments were calculated by measuring the absolute mean angular difference of the five exits preceding and the five exits following the treatment. In conditions where the animal displayed bimodal distribution of exit angles, angles were projected back onto the semi-circle surrounding the direction of most exit angles. A Mann-Whitney rank-sum test was used to determine if absolute angular difference between a treatment was significantly higher in the *test* condition (position of stimulus is changed by 90° between treatments) compared to the *control* condition (position of stimulus remains unchanged between treatments). The Mann-Whitney test was thus used to test if the animal turned with the stimulus. To test for homogeneity on two or more samples, a Mardia-Watson-Wheeler test was used. Generalised linear model (RStudio Team (2019). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA, <http://www.rstudio.com/>) was used to assess the relationship between degree of polarisation and probability of a turn (>45°).

Physiology

In preparation for intracellular recordings from the photoreceptors of dark-adapted individuals, the beetles were immobilized with beeswax and resin at a room temperature (for details see (Belušić et al., 2017) and mounted on a goniometric XYZ-stage that carried a micromanipulator (Sensapex, Oulu, Finland). A 50 μm diameter Ag/AgCl wire (inserted into the head capsule next to the eye) served as a reference electrode. Microelectrodes (Sutter, Novato, CA, USA) filled with 3 mol l⁻¹ KCl (resistance 100–150 M Ω) were inserted into the eye via a small triangular hole in the cornea, ventral of the

(expected) dorsal rim area. The signal was amplified using an SEC 10 LX amplifier (Npi electronic, Tamm, Germany) and a Cyber Amp 320 (Axon Instruments, Union City, CA, USA) and finally digitized via Micro 1401 (CED, Cambridge, UK). Spectral stimulation was provided with an LED array (“LED synth” (Belušič et al., 2017)), and with light from a Xenon arc lamp (XBO, Cairn, UK) filtered with a monochromator (B&M, Limburg, Germany). The light sources were tuned to emit equal numbers of photons at every wavelength (“isoquantal” mode). A UV transmissive polarisation filter (OUV2500, Knight Optical, UK) was mounted in a motorised rotator (Qioptiq, Germany) and inserted into the stimulation beam to facilitate measures of polarisation sensitivity. All cells were first quickly stimulated with the LED synth, to determine their spectral sensitivity within 2 s, after which their polarisation sensitivity was measured at their sensitivity peak (360 nm or 500 nm). This was followed by measuring the intensity-response function and a detailed spectral scan with a monochromator. The response amplitudes of single cells were transformed to sensitivities by means of an intensity–response function and a reverse Hill transformation (Belušič et al., 2017). Polarization sensitivity was calculated as the ratio between the sensitivity maximum and minimum, i.e., $PS = S_{max}/S_{min}$ (Bernard and Wehner, 1977). Some cells were lost during the spectral scan, hence N cells with measured polarisation sensitivity is higher than N cells with measured spectral sensitivity.

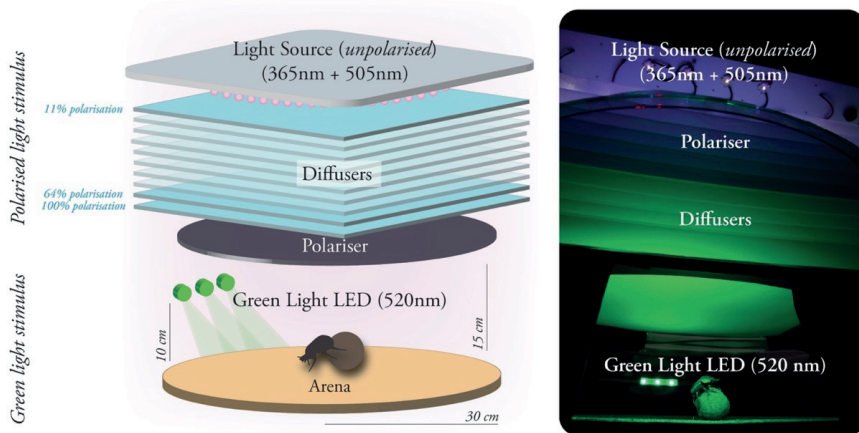


Figure 1. Description of the experimental setup. The experimental setup with an overhead *Polarised light stimulus* and a laterally presented *Green light stimulus*. The overhead light stimulus consisted of an unpolarised light fixture (‘Light Source’) of 80 UV light-emitting diodes (365 nm) and 21 cyan light-emitting diodes (510 nm) centred on a square shaped aluminium plate, along with ten sheets of ‘Diffusers’ (Plexiglas®), 1 cm apart, and a polarisation filter (‘Polariser’). Depending on the placement of the polarisation filter within the stack of diffuser, the degree of polarisation produced by the overhead light varied. The polariser could be placed in three different positions within the setup (highlighted in blue in the figure): *i*) before the ten sheets of Plexiglas (11 % polarisation), *ii*) before the 9th sheet (64 % polarisation), or *iii*) after the 10th sheet (100 % polarisation). The overhead light stimulus was suspended 15 cm above a circular arena of 60 cm diameter. The green unpolarised light stimulus (520 nm) consisted of 3 horizontally aligned LEDs (9.5 cm x 0.5 cm) presented to the beetle from either of the four sides of the arena (0°, 90°, 180° and 270°), 30 cm from the arena centre, at a height of 10 cm. Left image: schematic depiction of the experimental setup. Right image: real image of the experimental setup.

Light Measurements

Irradiance was measured by placing a cosine corrector coupled to a spectrometer via a calibrated light guide (cosine corrector: CC-3-UV-T; spectrometer: QE65000; light guide: P600-2-UV-VIS, Ocean Optics Inc., Dunedin, FL, USA) in the centre of the arena, 8 cm above the arena floor (corresponding to the position of the beetle on top of its dung ball) (Figure 1). *Degree of polarisation* of the light was analysed by a UV-transmissive linear polariser (Glan-Thompson; GTH5M-A: Thorlabs GmbH,

Dachau, Germany) coupled to a spectrometer via a light guide (spectrometer: FLAME-S-UV-VIS; light guide: P1000-2-UV-VIS; Ocean Optics). To avoid measuring off-axis light, the beam of light was sampled through an opaque lens tube (Foster et al., 2018).

Experimental Setup

The experimental setup consisted of *i*) an overhead polarised light stimulus, raised 15 cm above a flat, circular, sand painted 60 cm diameter arena, and *ii*) a green light stimulus presented from the side, 30 cm from the arena centre, at a height of 10 cm (*Figure 1*).

Polarised light stimulus

Having identified UV and green receptors with high polarisation sensitivity in the dorsal region of the dorsal eye of *Kheper lamarcki*, we decided to stimulate the DRA with a combination of UV and cyan light. 80 UV light-emitting diodes (LZ1-10UV00-0100; emission peak 365 nm, LedEngin Inc., San Jose, CA, U.S.A.) and 21 cyan light-emitting diodes (LXML-PE01-0070; emission peak 505 nm, Lumileds, San Jose, CA, U.S.A.) were mounted and arranged in a circular pattern (58 cm diameter) centred on a square shaped aluminium plate (60 x 60 x 0.2 cm), resting on a custom-built shelf mounted 50 cm above the arena floor. Ten sheets of Plexiglas® (60 x 60 x 0.3 cm, Plexiglas® Solar 2458, EBLA-GmbH, Appenweier, Germany), arranged in a stacked fashion, 1 cm apart, were placed 7.5 cm below the UV/cyan light fixture. Each sheet of Plexiglas was sand blasted on one side (facing downward) to act as a diffuser (Egri et al., 2016). A circular, UV-transmissive polarisation filter (BVO UV Polarizer, Bolder Vision Optik ©, Boulder, CO, USA; 60 cm diameter) was placed at three different positions within the setup; *i*) above the ten sheets of Plexiglas (*11 % polarisation*), *ii*) above the 9th sheet of Plexiglas (*64 % polarisation*), or *iii*) below the 10th sheet (*100 % polarisation*) (*Figure 1*). As a result of the experimental design, the animal was no less than 7-12 cm away from the overhead stimulus (see *Figure 1*). Thus, the overhead stimulus subtended a visual angle of approximately 136°-154° from the arena centre throughout all conditions. The combined polarised light stimulus had an irradiance of 1.26×10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$: cyan alone 2.39×10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$ and UV alone 1.04×10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$. This applied to all conditions where the polarised light stimulus was used, except for the condition in which the intensity of the polarised light stimulus was lowered. In this condition, the irradiance for 365 nm was lowered to 3.18×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$ while 505 nm remained unchanged.

Green light stimulus

The beetles were also presented with a green unpolarised light source (a previously documented replacement for the sun in the heading direction network of the beetle (el-Jundi et al., 2015)) consisting of 3 horizontally aligned LEDs (Adafruit DotStar Digital LED Strip; emission peak 520 nm, Adafruit Industries, New York, NY, U.S.A.). This ersatz sun (9.5 cm x 0.5 cm) was presented to the beetle from either of the four sides of the arena (0°, 90°, 180° and 270°) (*Figure 1*) at an intensity of 1.72×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$. When evaluating the isolated response to the ersatz sun, the polarised light stimulus was presented in the absence of a polariser.

Experimental method

A beetle was placed alongside its dung ball, in the centre of the circular arena and allowed to roll its ball to the perimeter where the exit bearing was noted. The beetle was then removed from its ball and placed back in the centre of the arena alongside its ball. This procedure was repeated five times. Beetles not successful in adhering to their bearing over their initial five exits ($p < 0.1$, Rayleigh uniformity test) were excluded from any further experiments.

Manipulation of directional input

Polarised light

Once the beetle had exited the arena five times, the polarisation filter was either kept in place (*control*) or turned by 90° (*test*) before the beetle was allowed to roll five additional times. The initial orientation of the filter alternated for each beetle, with every second beetle starting with the polarisation filter aligned to the 0°-180° direction of the circular arena, and every other beetle with the filter aligned perpendicular to this.

Ersatz sun

The initial position of the ersatz sun was placed in one of four positions around the arena (0°, 90°, 180° or 270°). Once the beetle had exited the arena five times with the ersatz sun in a fixed position, the apparent position of this light was either held stationary or changed by 90°, in relation to its previous position, before the beetle was allowed to roll five additional times.

Results

Ball-rolling dung beetles can orient to a green light stimulus

When the position of the ersatz sun was changed by 90° between two trials (*test*), the beetles changed their headings accordingly ($\mu = 93.55^\circ \pm 25.97^\circ$, $N = 15$, *Figure 2a*), with significantly larger turning angle compared to the *control* condition when the ersatz sun remained stationary ($\mu = 14.76^\circ \pm 9.77^\circ$, $N = 15$, *Figure 2a*, grey dotted line) (Mann-Whitney rank sum test, $W = 345$, $p < 0.001$, $z = 4.65$, $N = 15$). This clearly demonstrates that *Kheper lamarcki* can steer with reference to the green light source provided in the experimental arena.

Ball-rolling dung beetles orient with the same precision under a wide range of degrees of overhead polarisation

Intracellular photoreceptor recordings in the dorsal region of the dorsal eye of *Kheper lamarcki* revealed two types of spectrally distinct, but highly polarisation sensitive photoreceptors: one sensitive in the ultraviolet (UV, $\lambda_{\max} \approx 350$ nm) and one in the green ($\lambda_{\max} \approx 500$ nm) range of the electromagnetic spectrum (*Figure 3a*). Both photoreceptor types had high or very high polarisation sensitivities ($PS_{UV} = [3, 6, 25, 71]$; $PS_{UV}(\mu \pm \sigma) = 26.3 \pm 31.4$; $PS_G = [4, 4, 11.6, 8.3, 4.4, 4.12]$; $PS_G(\mu \pm \sigma) = 6.1 \pm 3.2$) (*Figure 3b*).

When the artificial, overhead band of polarised light (365 nm and 505 nm) was turned by 90°, the beetles turned in accordance with this turn under all three grades of polarisation presented (100% polarisation: $\mu = 88.74^\circ \pm 19.35^\circ$; 64% polarisation: $\mu = 72.80^\circ \pm 23.33^\circ$; 11% polarisation: $\mu = 67.96^\circ \pm 38.90^\circ$, $N = 15$) (*Figure 2b-d*). This turning angle differed significantly from when instead presented with the artificial band of polarisation in the same position for two consecutive trials (*control*) (100%: $\mu = 16.57^\circ \pm 10.01^\circ$; 64%: $\mu = 16.56^\circ \pm 8.73^\circ$; 11%: $\mu = 17.79^\circ \pm 14.87^\circ$) (*Figure 2b-d*, grey dotted line) (Mann-Whitney rank sum test, 100%: $W = 345$, $p < 0.001$, $z = 4.65$; 64%: $W = 340$, $p < 0.001$, $z = 4.43$; 11%: $W = 307$, $p = 0.002$, $z = 3.07$, $N = 15$). Although no significant difference in response could be found between the three conditions for neither test or control conditions (*control*: $p = 0.17$; *test*: $p = 0.69$, Mardia Watson Wheeler test, $N = 15$), the data shows a significant correlation between the degree of polarisation and the probability of a turn ($>45^\circ$), demonstrating that turning probability increases

with increasing degree of polarisation (GLM, $z = 2.23$, $AIC = 36.969$, $p = 0.0257$) (Supplementary Figure 1).

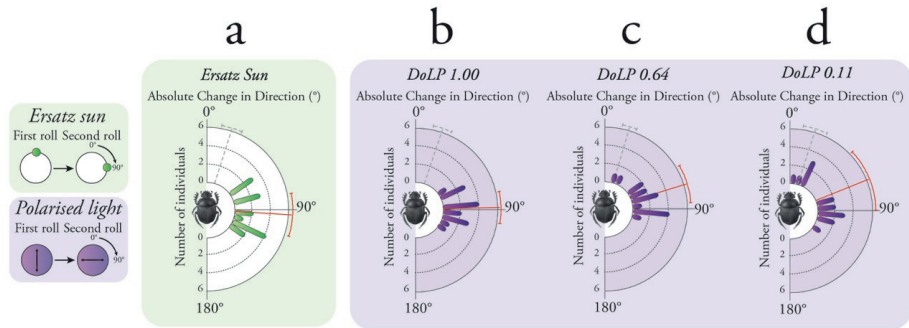


Figure 2. Response to directional change of compass cues. Kheper lamarcki was allowed to roll its dung-ball from the centre of a 60 cm diameter arena (a) presented with a lateral green light source (ersatz sun) in the presence of an overhead unpolarised light source, or (b-d) in the presence of a single overhead polarised light source (b: 100% polarisation; c: 64% polarisation; d: 11% polarisation). Once the beetle had reached the periphery of the arena, it was removed from its dung-ball and placed back in the centre alongside its ball. This procedure was repeated five times. After the fifth exit from the arena, the apparent position of the ersatz sun (a) or the e-vector direction of the artificial band of the overhead polarised light source (b-d) was turned by 90° (test), or remained in position (control). The beetle was then allowed to exit the arena again for five consecutive rolls. The absolute angular change between the mean direction of the five rolls prior to the treatment and the mean direction of the five rolls preceding the treatment in the test condition is depicted as coloured bars in all graphs. Under all four conditions, *K. lamarcki* changed bearing direction in accordance with the 90° angular change of the stimulus presented (red vector, all graphs). The absolute angular difference between the mean direction of the five rolls prior to the treatment and the five rolls preceding the treatment during the control condition is represented by a grey dotted vector in each graph. Error bars represent one circular standard deviation.

The weighting relationship between the ersatz sun and polarised light is highly dynamic

To investigate the weighting relationship of directional information from the sun (here represented by an ersatz sun) and the directional information from polarised skylight (here represented as an overhead polarised light source), the beetles were presented with both cues at the same time. When the ersatz sun was changed by 90° between trials (*test*), the beetles turned significantly in accordance with this change only when the degree of the polarised light presented from above (that remained in place) was set to its lowest setting of 11% polarisation (*control*: $\mu = 16.32^\circ \pm 10.46^\circ$; *test*: $\mu = 77.13^\circ \pm 21.94^\circ$, Mann-Whitney rank sum test, $W = 345$, $p < 0.001$, $z = 4.64$, $N = 15$) (Figure 4c). When rolling under the highest degree of overhead polarised light (100% polarisation) the beetles maintained their original bearing, seemingly ignoring the 90° change of the azimuthal position of the ersatz sun (*control*: $\mu = 16.55^\circ \pm 8.66^\circ$; *test*: $\mu = 16.72^\circ \pm 9.25^\circ$, Mann-Whitney rank sum test, $W = 231$, $p = 0.97$, $z = -0.042$, $N = 15$) (Figure 4a). *K. lamarcki* thus steered in reference to the ersatz sun when presented together with a low degree of overhead polarisation and in reference to the e-vector direction of the polarised light when presented a high degree of polarisation. In the presence of a polarised light stimulus of 64% polarisation, the beetles again changed their bearings, but now to a lesser degree ($\mu = 40.01^\circ \pm 26.06^\circ$, $N = 15$) (Figure 4b). Together, these results suggest that the weighting relationship between directional information from the ersatz sun and the polarised light source changes with changing degree of polarised light.

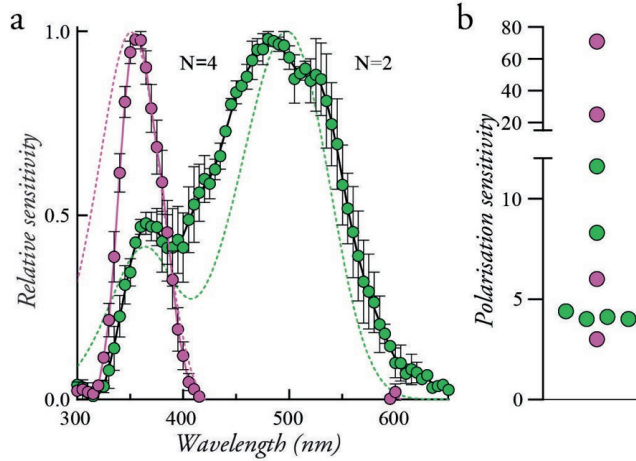


Figure 3. Intracellular photoreceptor recordings in the dorsal region of the dorsal eye of *Kheper lamarcki*. Intracellular recordings in the dorsal region of the dorsal eye of *K. lamarcki* revealed two types of spectrally distinct, but highly polarisation sensitive photoreceptors. (a) Spectral sensitivity of the ultraviolet sensitive photoreceptors ($\lambda_{\max} \approx 350$ nm) (pink graph) and the green sensitive photoreceptors ($\lambda_{\max} \approx 500$ nm) (green graph) in the dorsal region of the dorsal eye of *K. lamarcki*, fitted with rhodopsin nomograms (pink dashed line: $\lambda_{\max} = 352$ nm; green dashed line: $\lambda_{\max} = 501$ nm). (b) Polarisation sensitivities of ultraviolet sensitive (pink circles) and green sensitive (green circles) photoreceptors in the dorsal region of the dorsal eye of *K. lamarcki*.

The light intensity of the polarised light cue influences the weighting relationship

Given that the beetles neither conclusively maintained their original bearing, nor turned in accordance with the 90° azimuthal change of the ersatz sun when the overhead light was 64% polarised, we next lowered the intensity of the UV light of the polarised light approximately hundredfold (from 1.04×10^{15} to 3.18×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$). This allowed us to investigate if also the *intensity* of the polarised light would influence the weighting relationship between the two sources of directional information.

To confirm that the beetles were still able to respond to the e-vector rotation of this dimmer stimulus, we first presented the overhead light cue in isolation; either stationary (control: $\mu = 17.57^\circ \pm 13.64^\circ$, $N = 15$) or with a 90° rotation between trials ($\mu = 81.21^\circ \pm 14.98^\circ$, $N = 15$) (Figure 4e), i.e the beetles still turned in accordance with the turn of the polarisation axis of the overhead light (Mann-Whitney rank sum test, $W = 345$, $p < 0.001$, $z = 4.65$, $N = 15$). We further found that there was no significant difference in orientation performance between the groups of beetles orienting under the high and low intensity of the polarised light stimulus. This held true for both the control and the test conditions (control: $p = 0.22$, $W = 3.02$; test: $p = 0.39$, $W = 1.87$, Mardia Watson Wheeler test, $N = 15$).

When presented with the ersatz sun in combination with the lower intensity overhead polarised light, the beetles now turned in accordance with the positional change of the ersatz sun (control: $\mu = 16.78^\circ \pm 14.58^\circ$; test: $\mu = 80.20^\circ \pm 32.22^\circ$, $N = 15$) (Figure 4d) (Mann-Whitney rank sum test, $W = 340$, $p < 0.001$, $z = 4.44$, $N = 15$). This response was significantly different to the observed response when presented with an ersatz sun in combination with the full intensity polarisation stimulus of 64% ($p = 0.026$, $W = 7.28$, Mardia Watson Wheeler test, $N = 15$). This indicates that when the light intensity of the polarised light source was lowered, the weighting relationship between the two sources of information shifted towards directional information from the ersatz sun.

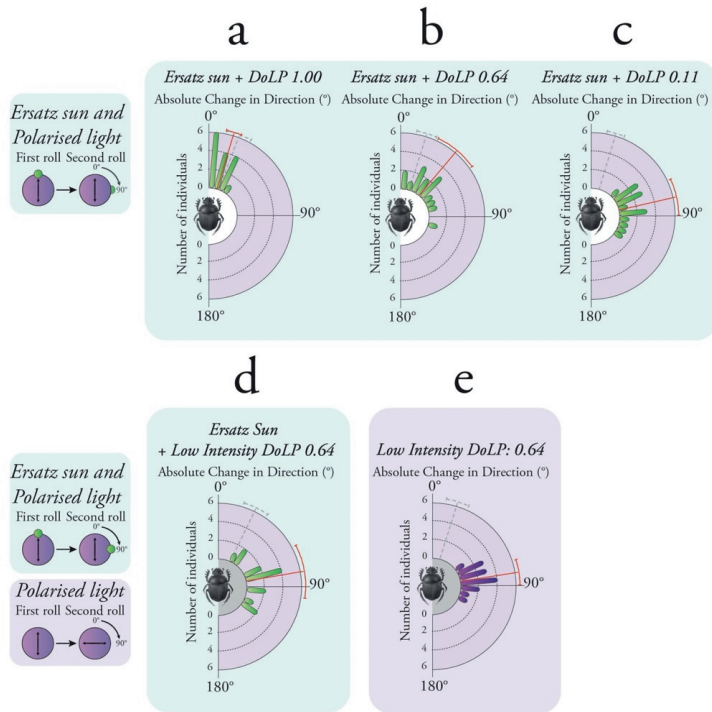


Figure 4. Response to directional change of the ersatz sun in the presence of polarised light. *Kheper lamarcki* was allowed to roll its dung-ball from the centre of a 60 cm diameter arena in the presence of a laterally presented green light source (ersatz sun) in combination with an overhead polarised light (a: ersatz sun in the presence of 100% polarisation; b: ersatz sun in the presence of 64% polarisation; c: ersatz sun in the presence of 11% polarisation; d: ersatz sun in the presence of 64% polarisation of lower UV light intensity), or (e) in the presence of a single overhead polarised light source of 64% polarisation of lower UV light intensity. Once the beetle had reached the periphery of the arena, it was removed from its dung-ball and placed back in the centre alongside its ball. This procedure was repeated five times. After the fifth exit from the arena, the apparent position of the ersatz sun (a-d) or the e-vector direction of the artificial band of the overhead polarised light source (e) was turned by 90° (*test*), or remained in position (*control*). The beetle was then allowed to exit the arena again for five consecutive rolls. The absolute angular change between the mean direction of the five rolls prior to the treatment and the mean direction of the five rolls preceding the treatment during the *test* condition is depicted as coloured bars in all graphs. When rolling in the presence of an ersatz sun under 100% polarised light, *K. lamarcki* did not respond to the positional change of the ersatz sun (a). In contrast, when rolling in the presence of an ersatz sun under 11% polarised light, *K. lamarcki* changed its bearing in accordance with the 90° angular turn of the ersatz sun (c). If instead presented with an ersatz sun in the presence of 64% polarised light, the beetles showed an intermediate response to the azimuthal change of the stimulus (b). However, when the intensity of the 64% polarised light decreased by hundredfold, the beetles again turned in response to the 90° turn of the ersatz sun (d). The absolute mean angular difference between the five rolls prior to the treatment and the five rolls preceding the treatment during the *control* condition is represented by a grey dotted vector in each graph. Error bars represent one circular standard deviation.

Discussion

*Evidence of UV and green polarisation sensitive photoreceptors in the dorsal rim area of *Kheper lamarcki**

Under a clear, sun-lit sky, the celestial polarised light pattern is highly distinguishable across all wavelengths of light. Under overcast skies or a tree canopy, the detection of this celestial pattern is most advantageous in the UV range (Barta and Horváth, 2004; Hegedüs et al., 2007; Seliger et al., 1994; Wang et al., 2014). Perhaps it is because of this stability that most insects, including honeybees (Labhart, 1980), ants (Duelli and Wehner, 1973), earth-boring beetles (Frantsevich et al., 1977), butterflies (Stalleicken et al., 2006) and flies (Hardie et al., 1979) analyse this pattern through UV sensitive photoreceptors. The unusually high polarisation sensitivity of 71 presented here for *K. lamarcki* in the UV (Figure 3) are very likely a result of electrical inhibition in the photoreceptor cell (Weir et al., 2016) or possibly due to mutual filtering in the fused rhabdom between orthogonally oriented rhabdomeres (Heras and Laughlin, 2017).

Interestingly, for the diurnal dung beetle *K. lamarcki*, our findings show evidence for polarisation sensitive photoreceptors in UV as well as green sensitive cells (Figure 3). Furthermore, the rare finding of two spectrally distinct, highly polarisation-sensitive photoreceptor classes (UV and green) for polarisation detection has also been suggested in the closely related, homing dung beetle, *Pachysoma striatum* (Dacke et al., 2002). *K. lamarcki* (as well as *P. striatum*) are active in open, dry habitats (Scholtz and Ranwashe, 2020), where the sky is clear and the degree of polarisation is very high (Brines and Gould, 1982; Horváth et al., 2014). In such conditions, the addition of green polarisation sensitive cells could perhaps increase the overall polarisation sensitivity of the animal's eyes, much as has been suggested in nocturnal insects (Belušič et al., 2017; Eggers and Gewecke, 1993; Labhart et al., 1992). However, for now, we can only speculate on this matter, as the current literature cannot answer why there are differences in wavelength sensitivities for polarisation detection in species across or within a family.

Response to the polarised light cue information as a function of its degree

When exiting the arena in the presence of an overhead polarised light source, presented in isolation, *Kheper lamarcki* showed a clear response to the 90° rotation of the artificial band of polarised light under 11%, 64% and 100% polarisation (Figure 2b-d). In addition, the probability of turning response (number of individuals that turn by 45° or more) decreased with decreasing degree of polarisation (Figure X), demonstrating a strong correlation between the degree of polarisation and turning response. The degree of polarised light is determined by the intensity of the electric field component in proportion to the light beam's overall intensity (Strutt, 1871; Suhai and Horváth, 2004) and can therefore act as a measure of signal strength: the higher the degree of polarisation, the stronger the signal. In crickets, the polarotactic response diminishes as the animal is presented a stimulus of lower degree of polarisation (Henze and Labhart, 2007; Labhart, 1996). If response to polarisation is limited by receptor noise (Labhart, 1996), then a greater signal strength would lead to more polarisation sensitive neurons being stimulated, thus a high degree of polarised light is likely to generate a stronger output signal and further affect the weighting strategy of the beetle's heading direction network. This can also be observed in nature; during overcast conditions, when the degree of polarisation is severely diminished (Barta and Horváth, 2004; Horváth et al., 2014), and therefore no longer a reliable directional cue, the ability to maintain a straight rolling bearing is disrupted in diurnal and nocturnal dung beetles alike (Dacke et al., 2013b; Dacke et al., 2013a). A similar correlation is also found in the nocturnal ball rolling dung beetle, *Scarabaeus satyrus* (Foster et al., 2019); when allowed to roll underneath an overhead polarised light source (similar to the polarised light source presented in this paper; Figure 1) of differing degrees of

polarised light, the ability of the beetle to maintain its exit bearing over consecutive rolls (orientation precision) lowered in correspondence to each degree of overhead polarised light presented.

The intensity of the polarised light cue affects its reliability as a directional cue

In this study, we find that *K. lamarcki* can reliably extract and utilise directional information from as low degree as 11% (Figure 2c), corresponding to the threshold limit suggested for its nocturnal cousin, *S. satyrus* (Foster et al., 2019). Coerced to roll during a moon-lit night, with the apparent position of the real moon covered from the beetle's field of view, the diurnal *K. lamarcki* do however fail to maintain a straight bearing (Smolka et al., 2016). It is important to note that the light intensity presented to the diurnal beetle in this study is three to four orders of magnitude higher than that presented to *S. satyrus* in Foster et al. 2019, and nearly six orders of magnitude higher than the intensity of polarised light in the night sky (Foster et al., 2019; Johnsen et al., 2006). Insects that carry an 'e-vector map' (a neural map of the e-vector distribution across the sky relative to the position of the sun) could, at least in theory, rely solely on the *direction* of the e-vector of the polarised light for directional information (Brines and Gould, 1979; Brines and Gould, 1982; Labhart, 1988; Labhart, 1996; Rossel and Wehner, 1984). Only when the noise of the visual signal outcompetes the difference between the orthogonally arranged groups of microvillar rhabdomeres, does the intensity of the polarisation cue become an important factor (el-Jundi and Homberg, 2012). Thus, the inability of *K. lamarcki* to steer straight according to the polarisation pattern surrounding the moon is very likely due to the limitations of the animal's own sensory ecology; the eyes of *K. lamarcki* might just not be able to detect the polarised skylight pattern (or any other additional celestial cues) across the night sky. For a future study, it would be of interest to find the threshold limit of polarised light detection in *K. lamarcki*, both in terms of degree of polarisation and intensity.

Varying the reliability of the polarised light cue influences the relative weighting relationship

When presented with an ersatz sun in combination with an overhead polarised light source at 11% polarisation, all beetles turned in response to the azimuthal displacement of the ersatz sun (Figure 4c). However, when presented in isolation, *K. lamarcki* is fully able to extract directional information from the weakly polarised light (Figure 2c). We interpret this relative weighting of directional information, now in favour for the ersatz sun, as if this single bright light generates a stronger and more reliable directional signal relative to the artificial band of polarised light. This weighting relationship is directly comparable to that observed outdoors; when the apparent position of the sun is changed by 180° with the aid of a mirror, while simultaneously blocking the real sun from view under a natural sky, *K. lamarcki* will turn in response to the mirrored sun (Dacke et al., 2014; Khaldy et al., 2019a; Khaldy et al., 2019b). This means that the directional information from the sun dominates in its heading direction network, not only over the celestial polarisation information, but over all remaining skylight cues. However, with the apparent position of the sun shaded from view, which can occur naturally by cloud cover or experimentally by the use of a shading board, these beetles instead follow the polarised light of the diurnal sky (el-Jundi et al., 2014). Now, the distribution of the relative weight between the directional cues that remain, shift in favour of the polarised light input.

When instead presented with a fully (100%) polarised light source, in addition to the same laterally presented ersatz sun as above, the beetle no longer turned in response to a 90° azimuthal change of the ersatz sun. Their consistent orientation along the same bearing was now instead guided by the stable e-vector direction of the overhead polarised light (Figure 4a). Thus, in this paradigm, directional information from the sun no longer dominated the heading direction network of the beetle, demonstrating that this species alters its weighting of cues in a context-dependant manner.

When next presented 64% polarised overhead light in combination with the ersatz sun, *K. lamarcki* rather changed their bearings by about 45° in response to the 90° rotation of the light (Figure 4b). Such

intermediate response, when two directional cues are set in conflict, can also be observed in ants (Lebhardt and Ronacher, 2014; Legge et al., 2014). With light polarised to 64% it consequently appears as if the two sources of input signals are providing directional information of similar reliability. But as soon as the intensity of the polarised UV light is lowered, the beetles again turn with the ersatz sun (*Figure 4d*). Showing that weakening the relative input of directional information from the polarisation cue effectively shifted the relative weighting towards the ersatz sun.

From the behavioural outcomes of our experiments, we can safely conclude that *K. lamarcki* integrates multiple sources of directional information in a Bayesian manner (Cheng et al., 2007; Körding, 2007; Körding and Wolpert, 2006), demonstrating clearly that directional information conveying the highest certainty at any given moment is afforded the greatest weight in the navigational network of the animal.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.K. and M.D.; Methodology: L.K., J.F., Y.G. and M.D.; Physiology: A.Y. and G. B.; Collection of animals: C.T. and M.B.; Data analysis: L.K., A.Y. and G. B.; Writing - original draft: L.K. and M. D.; Writing - review & editing: all authors; Funding acquisition: M.D.

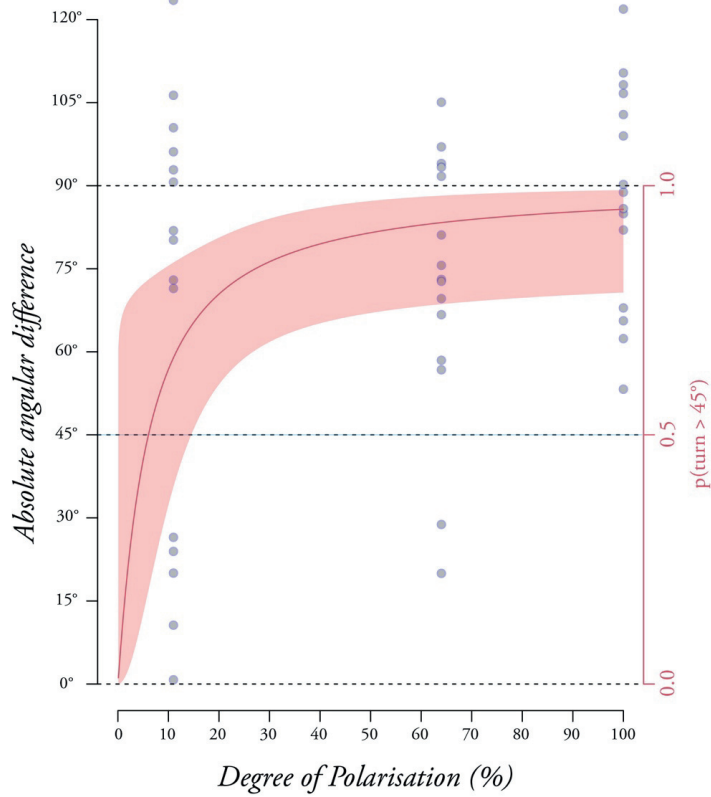
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Data availability

All data supporting reported results will be made available on zenodo.org prior to publication.

Supplementary Data




Supplementary figure 1. A logistic regression fitted to the probability of a turn larger than 45° when the stimulus was turned by 90°. There was a significant increase in the probability of a turn with increasing degree of polarization (11% polarisation: 10/15 individuals; 64% polarisation: 13/15 individuals; 100% polarisation: 15/15). We modelled this relationship as a linear increase in the log-odds of a turn with the base 10 logarithm of degree of polarization in percent (following Foster et al., 2019). The fitted model is shown as a red line with red shaded 95% confidence intervals, superimposed on the original turn angles.

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