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Using virtual reality to study visual performances of honeybees

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Virtual reality (VR) offers an appealing experimental framework for studying visual performances of insects under highly controlled conditions. In the case of the honeybee *Apis mellifera*, this possibility may fill the gap between behavioural analyses in free-flight and cellular analyses in the laboratory. Using automated, computer-controlled systems, it is possible to generate virtual stimuli or even entire environments that can be modified to test hypotheses on bee visual behaviour. The bee itself can remain tethered in place, making it possible to record neural activity while the bees is performing behavioural tasks. Recent studies have examined visual navigation and attentional processes in VR on flying or walking tethered bees, but experimental paradigms for examining visual learning and memory are only just emerging. Behavioural performances of bees under current experimental conditions are often lower in VR than in natural environments, but further improvements on current experimental protocols seem possible. Here we discuss current developments and conclude that it is essential to tailor the specifications of the VR simulation to the visual processing of honeybees to improve the success of this research endeavour.

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Introduction

Honeybees (*Apis mellifera*) constitute a privileged model system for the study of perception, learning and memory [1–5]. Despite their relatively small brain size, their perceptual and learning abilities are impressive. Foraging bees are able to navigate in complex environments, in which they can locate and repeatedly visit profitable food sources such as flowers [6]. Fundamental to these performances is their ability to associate certain environmental cues with food reward, namely the nectar or pollen found in flowers. In freely-flying bees, visually-driven performances of varying degrees of plasticity have been studied in controlled and carefully designed experiments in which a variety of sensory cues has been paired with a reward of sucrose solution [5]. In such free-flight conditions, it was possible to glean insights into sensory processing mechanisms. For example, honeybees, which possess trichromatic colour-vision [7], are colour-blind for visual tasks that involve edge-detection or motion sensing, as they rely on the exclusive sensory input to long-wave photoreceptors for these tasks [8]. Further, bees use the apparent image speed across the retina to perceive their distance from a visual cue [9]. However, in the visual domain, detailed investigations into the neural correlates of such interesting behaviours and processes have been stalled until quite recently, largely due to the absence of an experimental procedure in which bees are immobilised but perform sufficiently well on visual tasks. The same problem exists for the study of visual learning: while freely-flying bees learn to efficiently solve simple as well as complex discrimination problems [5], the neural underpinnings of these performances have remained elusive. Immobilisation of the bee remains essential for the application of most current invasive methods for recording neural activity, even in today's age of technological miniaturisation.

A first attempt to study visual associative learning in immobilised bees made use of the proboscis extension response (PER), a reflexive, appetitive behaviour exhibited by hungry bees when sucrose solution and other sweet tastants contact their antennae, tarsi and/or mouthpieces [10,11]. Makoto Kuwabara was the first to report visual conditioning of PER using chromatic lights paired with sucrose solution delivered to the tarsi [12]. Yet, since then, and despite repeated attempts, learning rates and discrimination capabilities as revealed by this protocol remain low and far from those of freely-flying bees (see review in [13]).

Novel attempts were therefore developed and among them, virtual reality (VR, see Box 1) offers an appealing experimental framework to overcome these limitations. It refers to a simulated environment, perceived and updated by the actions of an animal immersed in the simulation

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Box 1 Virtual reality for insects — a potted history.

An early predecessor of a VR system for walking insects was published more than 60 years ago by Bernhard Hassenstein to characterise for the first time optomotor responses in insects [46]. In this setup, a tethered beetle held onto a very lightweight 'Y-maze globe' made of thin straws. This ball turned below the beetle as the beetle ' walked' along a blade of straw, thus repeatedly confronting the beetle with Y-maze choices of diverging straws. The tethered beetle could then be exposed to highly controlled, moving visual stimuli, simultaneously recording its directional choices on the globe [47]. Following designs, so-called locomotion compensators, then allowed for less constrained movements of the insect on two-dimensional surfaces (flies: [48], silkmoths: [49]). These early setups were technically demanding, and involved insects walking unrestrained (untethered) on top of a rolling ball. The movement of the insect on the ball is constantly monitored and recorded, and servo-motors at the side or bottom of the ball compensate for this locomotion by moving the ball in opposite directions. The insect therefore always remains at the apex of the ball, where it is presented with controlled olfactory or visual stimuli. Locomotion compensators have been very successful for behavioural analyses [50]. For simultaneous neuronal recordings, the insect itself is immobilised by a tether holding it in place on top of a lightweight trackball, which is suspended freely on an airflow. The walking movements of the insect are thus directly translated into ball movements, which can be recorded with precision and also used to directly manipulate the presented cues in real-time (closed-loop). While walking VR setups have been used for investigating olfactory cues (e.g. in bees: [20,51,52]) or acoustic cues (e.g. in crickets: [50]), they are particularly useful for the presentation of visual cues. Screens consisting of LED bulb arrays are commonly employed (e.g. [21••,53,54]), but projection-based designs have also been developed more recently (see [39•] for an example in spiders). Trackball setups can be profitably used in natural visual surroundings too [55•].

[14]. Using automated, computer-controlled systems, it is possible to generate abstract or realistic virtual stimuli and even entire landscape displays that can be modified to test specific hypotheses on visual behaviour. This approach offers, therefore, a valuable compromise between a controlled experimental environment and an ecologically rich surrounding in which an individual animal can be studied [15]. Here we will focus on discussing some recent attempts to study honeybee visual behaviour in virtual reality environments. This may help to overcome the limitations of using only free-flying bees to study visually-driven behaviour and may stimulate further efforts in this direction.

Bees in virtual reality: studying navigation and attentional processes

A first important breakthrough was achieved by Luu *et al.* [16], who placed a tethered bee in the middle of a setup of four LCD monitors that displayed a moving panorama (Figure 1a). The goal was to study how passive image motion affects the behaviour of a flying bee *en route* to the goal. The authors were able to make the tethered bees fly in these experimental conditions and noticed that, upon such suspended flight, bees slightly raise their abdomen, a response that is interpreted as a 'streamlining response', presumably to reduce aerodynamic drag. This response is elicited by pure visual exposure ([16]; Figure 1b,c) and is strongest when the image motion is in the direction that would be experienced during forward flight and when it covers the full visual panorama of the bee. It shows highest sensitivity in the lateral rather than in the frontal and rear fields, and is also modulated by air-flow cues simulating head-wind [17].

An alternative to bees flying stationary is the study of tethered bees walking on top of a light-weight trackball suspended on an airflow. Ball movements can be tracked accurately by appropriate optical mouse sensors [18] or a video camera [19]. This kind of device, usually termed locomotion compensator, running sphere or treadmill, has been used since more than four decades to study different aspects of insect behaviour, in particular stereotyped responses to environmental stimuli [20]. Yet, the coupling with a visual environment that is directly updated by the movements of the insect walking stationary (closed-loop) represents a novelty. Paulk *et al.* [21••] used a variant of such a closed-loop VR setup for studying attention-like processes in tethered walking honeybees. Bees walking stationary in the middle of a LED arena (Figure 1d) were presented with one (Figure 1e) or two competing green vertical bars separated by 90° (Figure 1f) and flickering at different frequencies. The authors were able to combine the recording of behavioural fixation of these stimuli, reflecting attention, with an electrophysiological analysis of neural activity in different parts of the bee brain, inspired by earlier work on *Drosophila* [22]. In this way, neural responses to a specific stimulus could be 'frequency tagged' and thus traced in measurements of local field potentials, showing that attention-like processes occur in the optic lobes before the bee displayed a behavioural choice. The use of closed-loop instead of open-loop controlled visual stimuli seems to be an important parameter, as it increases the temporal coordination of neural activity in the insect brain [23•]. In a follow-up study, van de Poll *et al.* [24••] focussed on a detailed exploration of choice behaviour of honeybees. Tethered bees were again placed on a trackball and surrounded by a hexagonal LED arena. The LED screens presented two or more vertical green bars that differed in their visual flicker frequency. The bee had closed-loop control over the stimulus movements, and could spontaneously choose among the presented stimuli through fixation. The authors were able to show that honeybees payed more attention to fixated bars flickering at 20–25 Hz, while they avoided higher or lower frequencies (50–100 Hz and 2–4 Hz, respectively).

The VR setups used across these studies are constantly evolving, and each study so far has used slightly different parameters and materials (see also Box 1). Most importantly, the techniques used for visual stimulus presentation have changed from LCD to LED screens, as LCD screens do not allow for easy control over parameters such

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