Floor and ceiling mirror configurations to study altitude control in honeybees

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Julien R. Serres[1*†], **Antoine H.P. Morice**[1†], **Constance Blary**[1,2‡], **Romain Miot**[1,3‡], **Gilles Montagne**[1†], **Franck Ruffier**[1†]

***For correspondence:** julien.serres@univ-amu.fr (JRS)

[†]These authors contributed equally to this work
[‡]These authors also contributed equally to this work

[1]Aix Marseille Univ, CNRS, ISM, Marseille, France; [2]CEFE UMR 5175, CNRS - Université de Montpellier - Université Paul-Valéry Montpellier - EPHE - 1919 route de Mende, 34293 Montpellier cedex 5, France; [3]XTIM SAS, 77 rue de Lyon, 13015 Marseille, France

Abstract To investigate altitude control in honeybees, an optical configuration was designed to manipulate or cancel the optic flow. It has been widely accepted that honeybees rely on the optic flow generated by the ground to control their altitude. Here, we create an optical configuration enabling a better understanding of the mechanism of altitude control in honeybees. This optical configuration aims to mimic some of the conditions that honeybees experience over a natural water body. An optical manipulation, based on a pair of opposed horizontal mirrors, was designed to remove any visual information coming from the floor and ceiling. Such an optical manipulation allowed us to get closer the seminal experiment of Heran & Lindauer (1963). Our results confirmed that a reduction or an absence of ventral optic flow in honeybees leads to a loss in altitude, and eventually a collision with the floor.

Introduction

Flying bees, honeybees or bumblebees, are known to be particularly sensitive to the optic flow pattern generated by the contrasting features of the ground to adjust their altitude by maintaining constant ventral optic flow during terrain following tasks (***Baird et al., 2006***; ***Portelli et al., 2010b***; ***Srinivasan, 2011***; ***Portelli et al., 2017***; ***Serres and Ruffier, 2017***; ***Lecoeur et al., 2019***). The minimum sized section of a rectangular tunnel sets the honeybees' forward speed (***Portelli et al., 2011***; ***Serres and Ruffier, 2017***), therefore the forward and upward axes of the honeybee's flight control system can be decoupled (***Portelli et al., 2010b***). Consequently, in a tunnel where the width is smaller than the height, the width sets the honeybees' forward speed, see also (***Franceschini et al., 2007***; ***Portelli et al., 2010a***; ***Raharijaona et al., 2017***; ***Bergantin et al., 2021***) for a description of the visuomotor modelling in honeybees. Similarly, honeybees, which have be trained to follow the tunnel ceiling, when encountering a "dorsal ditch" in the middle of the tunnel configuration (***Portelli et al., 2017***) responded to this new configuration by rising quickly and hugging the new, higher ceiling, while maintaining a similar forward speed, distance to the ceiling, and dorsal optic flow to those observed during the training step. Conversely, honeybees trained to follow the floor kept on following the floor regardless of the change in the ceiling's height (***Portelli et al., 2017***).

The present study aims to pursue investigations about the role of dorsal and ventral visual inputs feeding the altitude control system in honeybees by manipulating or cancelling parts of the optic flow using mirrors. Inspired by ***Duchon and Warren Jr*** (***2002***) experiments on humans, in which the researchers designed an optical manipulation made with a pair of infinite walls in or-

der to optically remove the floor, then by strongly decreasing visual information coming from the floor, we designed a novel optical configuration with floor and/or ceiling mirror(s) enabling a better understanding of the mechanism of altitude control in honeybees. Such an optical manipulation in which the floor appeared to be removed allowed us to mimic some of the conditions that honeybees experience when experimentally trained to fly over a natural water body and extend the seminal experiment of ***Heran and Lindauer*** (***1963***). Sixty years ago, these researchers trained honeybees to fly above a 247m-long water surface. When the water surface was rippled or when a floating bridge provided a visual contrast, honeybees were able to cross the lake. However, honeybees flying over a calm water surface during foraging trips flew lower and lower until they collided with the water surface and drowned (***Heran and Lindauer, 1963***). Our flight tunnel, which used a pair of mirrors placed on the floor and on the ceiling, gets closer to, and extends experimentally, a behaviour which has been observed outdoor above a water surface.

Methods and Materials

Flight tunnel

The outdoor flight tunnel is a rectangular shape (220 cm long, 71 cm high and 25 cm wide, see Fig. S1 for further details), the ceiling and floor are mirrors that can be covered as in Step 0 in Fig. S2, the left hand wall is solid and the right hand wall is made of insect netting. A unique red and white striped pattern, perpendicular to the long axis of the tunnel and therefore the direction of flight, is provided on the four surfaces of the tunnel (floor, ceiling, left wall, insect netting) although it is only permanently installed on walls. The mirrors on floor and ceiling can be covered with the same pattern as can the insect netting. In this latter case the stripes are reproduced using a red gelatin filter (Lee Filters HT019). The tunnel is closed with white boards at each end. At one end there is a circular entrance (5 cm in diameter) located 11.5 cm above the floor. At the other end, a square opening (3.5 cm) placed 11.5 cm above the floor gives the bees access to the reward box. The tunnel's entrance and the door of the reward box were opened and closed manually by the experimenter. The flight tunnel received only indirect illumination (no direct sunlight). This video can be watched to better understand the organization of our experimental set-up: https://youtu.be/KH9z8eqOBbU.

Pattern

Red stripes of two different widths (1 cm and 3 cm) form a simple 10 cm wide regularly repeated pattern. The angular subtends of the stripes ranged from 5.7° to 53° (1–10 cm-wide pattern viewed from a distance of 10 cm) and from 0.5° to 5.3° (1–10 cm-wide pattern viewed from 1 m). As honeybees do not possess red-sensitive photoreceptors (***Srinivasan, 2011***), they perceive red stripes as grey ones. Between the red and white stripes, the Michelson contrast is 0.47 but 0.25 on the insect netting. The contrast was measured using a photodiode equipped with a green band-pass filter (Kodak Wratten n°61), the transmission spectrum of which closely matched the spectral sensitivity of the honeybee's green receptors.

Experimental procedure

See Supplementary material S1.

Video recordings and flight path analysis

See Supplementary material S2.

Statistical analysis

See Supplementary material S3.

Results

Honeybees following the floor do not rely on dorsal visual information

In experiment A (Fig. 1A), we tested the effect of a visual impoverishment in the dorsal part of the honeybees' visual field. The chronology of the procedure from step 0 to step ii is described in Fig. S2. A group of 27 honeybees were trained in the control condition (CC) (Fig. 1Ai-Aii). The 1st flight "in mirror on the ceiling" condition, in which the ceiling mirror was uncovered, was recorded in an impoverished visual condition (IC) (See Supplementary material S1). The presence of the mirror on the ceiling appearing to double the tunnel's height (142cm) upwards (Fig. 1Aiii-Aiv). We observed no significant change in flight behaviour in honeybees (Exp. A in Table S1).

Without any ventral and dorsal visual information, honeybees collide with the floor mirror

In experiment B (Fig. 1B), we tested the effect of a visual impoverishment in both the dorsal and ventral parts of the honeybees' visual field. The chronology of the procedure is described in Fig. S2. A group of 15 honeybees were trained in the control condition (CC) (Fig. 1Bi-Bii). The 1st flight, "in double mirror" condition in which both mirrors were uncovered, was recorded in an impoverished visual condition (IC) (See Supplementary material S1). The presence of mirrors on the ceiling and on the floor created an optical manipulation in which a pair of infinite walls appeared. As a result, no visual information from either the floor or ceiling was available (Fig. 1Biii-Biv). We observed significant changes in flight behaviour in honeybees (Exp. B in Table S1) from x = 8cm until each of the honeybees collided with the floor mirror.

Dorsal visual information does not aid in flying further over the floor mirror

In experiment C (Fig. 1C), we tested the effect of a visual impoverishment in the ventral part of the honeybees' visual field. The chronology of the procedure is described in Fig. S2. A group of 15 honeybees were trained in the control condition (CC) (Fig. 1Ci-Cii). The 1st flight "in mirror on the floor" condition in which the floor mirror was uncovered, was recorded in impoverished visual condition (IC) (See Supplementary material S1). The presence of the mirror on the floor appearing to double the tunnel's height (142cm) downwards (Fig. 1Ciii-Civ) making a kind of "ventral ditch" of 71cm in depth. We observed significant changes in flight behaviour in honeybees (Exp. C in Table S1) from x = 40 cm until each of the honeybees collided with the floor mirror. Honeybees may be visually attracted to the virtual floor 71 cm below, but then they collided with the mirror.

Covering the first half of the floor mirror does not help honeybees to fly further

In experiment D (Fig. 1D), we tested the effect of a visual reinforcement in the ventral part of the honeybees' visual field by covering the first half of the floor mirror with the same pattern of red and white stripes as previously used. The chronology of the procedure is described in Fig. S2. A group of 14 honeybees were trained in the control condition (CC) (Fig. 1Di-Dii). The 1st flight "in half mirror on the floor" condition in which the second half of the floor mirror was left uncovered, virtually doubling the tunnel height (142 cm) downwards (Fig. 1Diii-Div) making a kind of "ventral ditch" of 71 cm in depth as in experiment C (Fig. 1C). We observed significant changes in flight behaviour in honeybees (Exp. D in Table S1) from x = 139 cm until each of honeybees collided with the mirror. This additional texture on the floor does not help honeybees to fly further above the mirror. The travelled distance above the mirror (39 cm) was similar to the one observed in experiment C (see Exp. C in Table S1).

Discussion and Conclusion

The aim of the present study was to extend ***Heran and Lindauer*** (***1963***) work by precisely manipulating parts of the optic flow. The use of a mirror on the floor (or ceiling) allowed us to manipulate the ventral (or dorsal) optic flow, thus generating a "ventral (or dorsal) ditch", without having to change

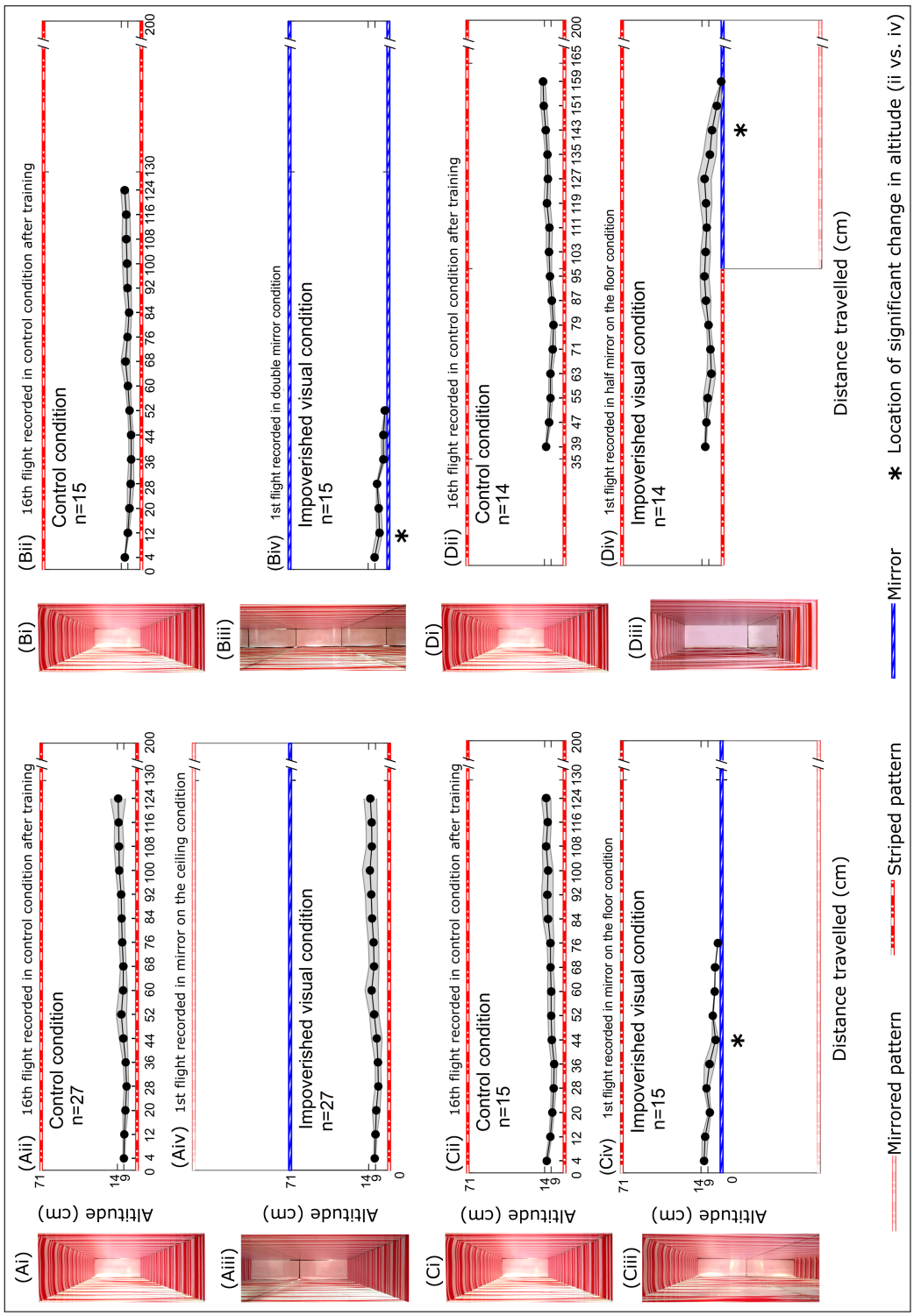

Figure 1. This figure depicts the trajectories (Median ± MAD) followed by honeybees in the vertical plane (*x*,*z*) in the four experimental conditions (Exp. A, Exp. B, Exp. C, and Exp. D). In each panel, a comparison can be made between the trajectories produced prior to (Aii, Bii, Cii and Dii) and after (Aiv, Biv, Civ and Div) the experimental manipulations. These manipulations consisted of uncovering either a mirror on the ceiling (Aiii), mirrors on both the floor and the ceiling (Biii), a mirror on the floor (Ciii) or on half the floor (Diii). High resolution pictures in the tunnel can be seen in ***Serres et al.*** (***2019a***). Results show that while manipulating dorsal optic flow does not affect the honeybees' trajectories, manipulating ventral optic flow, whether it is a simple manipulation or a suppression, has significant consequences on the trajectories, leading to systematic collisions with the floor mirror. The abscissa marked by a ∗ symbol in (Biv), (Civ), and (Div) represents the location at which we observed a significant change in height of flight before collisions (see Table S1 for comparisons of altitude distributions).

the orientation of the pattern stripes (***Baird et al., 2006***), the pattern relative velocity (***Portelli et al., 2010b***) or the tunnel geometry (***Portelli et al., 2017***). On the other hand, a double mirror condition allowed us to simultaneously suppress visual information coming from both the floor and ceiling, generating two infinite parallel walls.

In experiment A (Fig. 1Aiv), honeybees appear to follow the floor despite this virtual "dorsal ditch" because they may have learnt to follow the floor and to rely on the ventral visual information in order to regulate their flight, in harmony with ***Portelli et al.*** (***2017***) results. The lack of a difference between last training flight and test trial data in experiment A suggests that the upward change in tunnel height might simply not have been perceived by the honeybees.

Conversely, each experimental manipulation that affects the ventral part of the optic flow, whether it is a total deprivation of the ventral optic flow in experiment B (Fig. 1Biv) or a reduction in the ventral part of the optic flow (Figs. 1Civ,Div), gave rise to a loss of altitude until the honeybee collided with the floor mirror. Interestingly our double mirror condition allowed us to get closer to the flight conditions of an open sky flight above a calm water surface as used by ***Heran and Lindauer*** (***1963***). Our results agree with theirs insofar as the honeybees lose altitude in the absence of ventral optic flow.

In experiments C and D, the use of a mirror on the floor does not cause the suppression of the ventral part of optic flow, as the ceiling textures mirrored in the floor create a virtual "ventral gap" reducing the resulting ventral optic flow. The honeybees' reduction in altitude until they collide with the mirror could result from a change in altitude intended to restore the ventral optic flow as experienced in the 15 control trials, which is in harmony with both ***Portelli et al.*** (***2010b***) and ***Portelli et al.*** (***2017***) results. Taken together, all our results indicate unequivocally that the drowning observed in the study of ***Heran and Lindauer*** (***1963***) reflect the propensity of honeybees to reduce their altitude, in order to restore the ventral optic flow experienced.

Results in experiments C and D (Figs. 1Civ-Div) reveal that a significant proportion of honeybees (half of the sample, see Table S1) can fly as far as 39 cm above the floor mirror without losing altitude showing that they perceive the ventral optic flow over a wide visual field. By picking up information over a wide visual field (***Lecoeur et al., 2019***), honeybees detect and respond to changes in the environment even when lacking texture on the ground.

To conclude this study, there are different strategies of altitude control in insects. ***Straw et al.*** (***2010***) demonstrated that flies do not use ventral optic flow in their altitude control. Our findings could be expanded or be useful in other insect species to test whether they are sensitive to ventral optic flow, or if other optical information controls their altitude (***Berger Dauxère et al., 2021***). Our set-up could also be re-scaled to study the effect of optic flow on altitude control in birds (***Altshuler and Srinivasan, 2018***; ***Serres et al., 2019b***).

Acknowledgments

The authors would like to thank Marc Boyron and Julien Diperi for their technical assistance with designing the flight tunnel, Patrick Sainton for his video montage showing the experimental set-up, and they wish to thank David Wood (English at your Service, http://www.eays.eu/) for revising the English of the manuscript. We are grateful to the two anonymous reviewers, whose suggestions helped us improve the manuscript.

Funding

This work was supported by Aix Marseille University and the CNRS.

Data accessibility

The data that support the findings of this study are openly available in Supplementary Material section at https://www.biorxiv.org/content/biorxiv/early/2021/10/07/2021.09.23.461476/DC1/embed/media-1.zip?download=true.

Additionnal information

Author contributions

JRS, AHPM, GM, and FR designed the experiments. CB performed the experiments. RM developed the tracking app with Matlab software. JRS, AHPM, CB, GM, and FR analysed the data. JRS wrote the first draft of the paper; all authors prepared and revised the manuscript. JRS coordinated and supervised this research work.

Ethics

Animal experimentation: study involved experiments on honeybees and were conducted according to ethical guidelines.

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Supplementary material

Floor and ceiling mirror configurations to study altitude control in honeybees

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S1 Experimental procedure

During the experimental period, a small set of four groups comprising from 14 to 27 freely flying honeybees (*Apis mellifera*) were colour-marked and trained daily to enter and fly alone along a tunnel to collect a sugar solution reward (25% sucrose w/w) at the opposite end. Their flight path toward the reward was recorded with a digital camera from the insect-netting side, strictly in keeping with the chronology of the procedure depicted in Fig. S2 using a distinct group of honeybees for each of the four experimental conditions (Exp. A, Exp. B, Exp. C, and Exp. D).
In the first session (called "Constant floor-roof condition" - Control Condition CC), honeybees were trained over 16 trials to follow the ground (Fig. S2 A(0+i)-D(0+i)). In a second session (called "Conditions with mirror(s)" - Impoverished visual Conditions IC), one mirror, both mirrors, or the half of the floor mirror were uncovered (Fig. S2 A(ii)-D(ii)), appearing to double the tunnel's height when there was one (or half) mirror uncovered (above in Exp. A; below in Exp. B & D), or giving the appearance of an infinite tunnel height when there were two mirrors uncovered in Exp. C.

S2 Video recordings and flight path analysis

The honeybees' trajectories were filmed at a rate of 100 frames per second with a high-resolution black-and-white CMOS camera (Teledyne Dalsa Genie HM640). The video recording was manually triggered after the experimenter opened the tunnel entrance to the honeybees, who were sent through one by one. A red filter (Lee Filters HT019) was positioned in front of the camera monitoring the honeybee's traces. This process removes the red stripes from the trajectory records and optimizes the contrast between the honeybee and the background. The camera was placed at a distance of 2.3 m from the insect netting. The field of view (130 cm in width, 71 cm in height) covered the whole height of the tunnel, from abscissa x = 0 cm to abscissa x = 130 cm in experiments A-C, and from abscissa x = 35 cm to abscissa x = 165 cm in experiment D. Image sequences were calibrated, corrected, processed and analysed using a custom-made Matlab program available online (see: `https://github.com/rm1720/bees-applications/wiki`). This program automatically determined the honeybees' flight height z in each frame as a function of the abscissa x along the tunnel axis so that the honeybee's trajectory in the vertical plane could be plotted. Only trajectories until the first collision were plotted and analysed.

S3 Statistical analysis

All the data recorded were included in the statistical analysis without removing any outliers. Statistical data analyses were performed with the Matlab R2018a software program. Not all datasets exhibit the same number of measurements as a function of the abscissa because the bees collide with the floor mirror at different points along their trajectories. As a consequence, median and Median Absolute Deviation (Median ± MAD) values were computed for all datasets by binning each bee's trajectory with an 8 cm window in abscissa (see Table S1). Mann–Whitney U tests were used to compare altitude binning distribution by pairs: control conditions (CC) versus impoverished visual conditions (IC) in each experiment (Exp. A, Exp. B, Exp. C, and Exp. D, see Table S1).

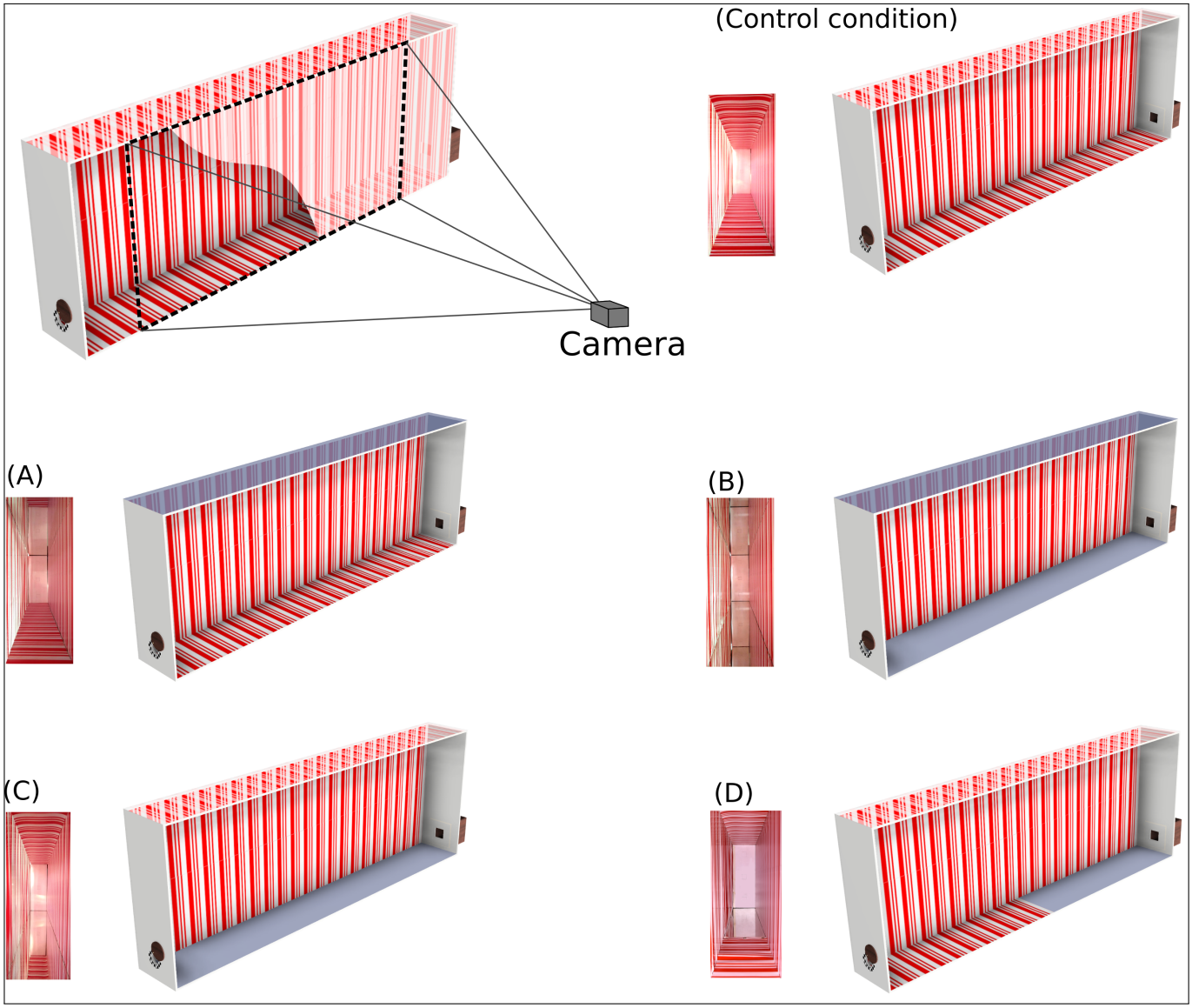

Figure S1. The flight tunnel is a rectangular shape (220 cm long,71 cm high and 25 cm wide. In the control condition (CC), the four surfaces of the tunnel were textured with red and white stripes oriented perpendicularly to the bees' flight paths. One side of the tunnel consists of insect netting lined with stripes. The camera was placed sideways, 2.3 m from the insect netting. The field of view (130 cm in width, 71 cm in height) covered the whole height of the tunnel, from abscissa x = 0 cm to abscissa x = 130 cm in experiments (A) , (B), and (C), and from abscissa x = 35 cm to abscissa x = 165 cm in experiment (D). In experiment (A), only the ceiling mirror was uncovered. In experiment (B), both mirrors were uncovered. In experiment (C), only the floor mirror was uncovered. In experiment (D), only the floor mirror was half uncovered.

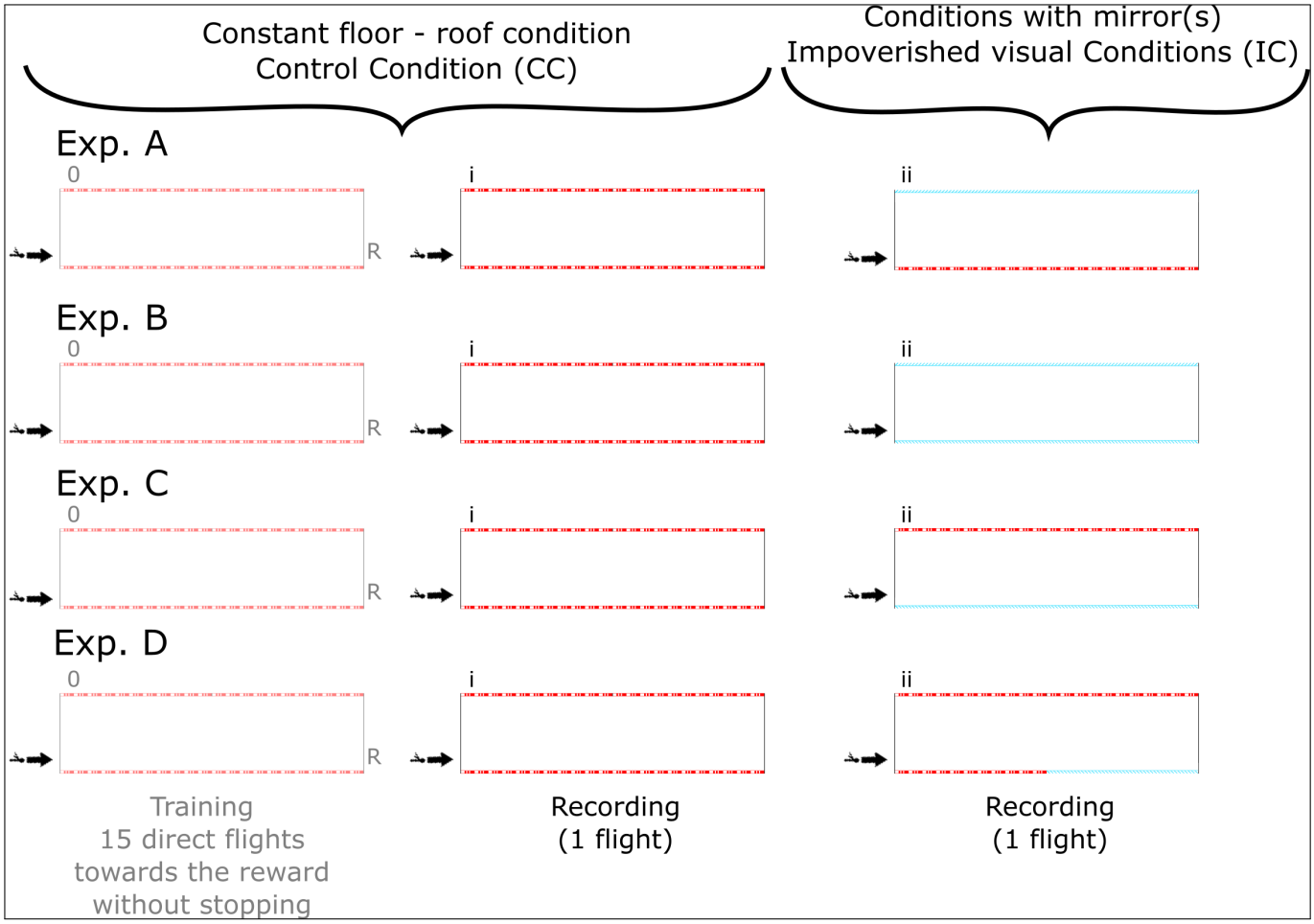

Figure S2. Chronology of the procedure used in the four experimental conditions (Exp. A, Exp. B, Exp. C, and Exp. D). The same individual bees were trained and tested and their trajectories were recorded in line with the strict chronology of the steps (0, i, and ii). The arrow represents the entrance position near the floor, and the letter 'R' represents the reward box location behind a door near the floor. The entrance and the door to the reward box 'R' are both located 11.5 cm above the floor.

Step 0 (the training step - 15 direct flights): the honeybees were first trained by completing 15 flights travelling along the tunnel with a uniform height of 71 cm (called "Constant floor-roof condition") to collect nectar in a reward box. Honeybees were rewarded at the end of each flight; the entrance to the reward box was closed during the flights so that no visual cues were available about the position of the reward, but the door was opened on arrival.

Step i (Control Condition CC, video-recording - 1 flight): immediately after the training step, individual bees' trajectories were recorded in this same tunnel with a uniform height of 71 cm (called "Constant floor-roof condition"); the entrance to the reward box was closed during the flights so that no visual cues were available about the position of the reward.

Step ii (Impoverished visual Conditions IC, video-recording - 1 flight): immediately after recording the bees' trajectories under "Constant floor-roof condition", one mirror (Exp. A and Exp. C), two mirrors (Exp. B), or the half floor mirror (Exp. D) were uncovered, and the bees' trajectories were recorded in the presence of this optical manipulation (either virtually doubling the tunnel height when there is one mirror uncovered, or giving virtually an infinite tunnel height when there are two mirrors); the entrance to the reward box was again closed during these flights so that no visual cues were available about the position of the reward.

Exp. A	Binning x (cm)	0-8	8-16	16-24	24-32	32-40	40-48	48-56	56-64	64-72	72-80	80-88	88-96	96-104	104-112	112-120	120-128
Control condition	Size N	13	27	27	27	26	26	27	27	27	25	25	24	23	23	23	22
	Median(z) (cm)	9.0	8.5	7.7	6.9	7.5	9.4	10.6	9.2	9.3	10.3	10.5	11.3	13.2	12.7	12.5	13.7
	MAD(z) (cm)	1.4	1.3	2.6	3.6	4.2	5.5	6.6	6.6	5.3	4.9	5.4	4.6	5.8	6.6	6.7	7.6
Impover -ished condition	Size N	13	26	27	26	25	26	25	24	24	24	24	24	24	24	24	23
	Median(z) (cm)	9.6	9.0	8.5	6.9	7.2	8.1	10.0	11.9	10.0	10.4	11.6	12.1	13.1	11.7	11.8	12.8
	MAD(z) (cm)	0.9	1.7	3.7	3.7	3.8	4.9	4.5	5.5	5.8	5.6	5.6	6.2	6.6	7.3	8.2	7.8
CC vs. IC	p-value	0.54	0.31	0.78	0.99	0.58	0.50	0.56	0.74	1	0.87	0.96	0.89	0.91	0.46	0.63	0.67
Exp. B	Binning x (cm)	0-8	8-16	16-24	24-32	32-40	40-48	48-56	56-64	64-72	72-80	80-88	88-96	96-104	104-112	112-120	120-128
Control condition	Size N	13	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
	Median(z) (cm)	11.2	9.0	7.8	7.0	6.6	6.6	7.8	9.0	10.8	9.3	8.1	9.3	9.6	10.2	10.2	11.4
	MAD(z) (cm)	0.7	1.2	2.3	2.4	2.0	1.7	1.9	2.5	3.4	3.5	3.6	4.1	4.2	3.7	3.9	4.3
Impover -ished condition	Size N	15	15	8	5	3	2	1	0	0	0	0	0	0	0	0	0
	Median(z) (cm)	9.2	4.6	5.2	7.3	2.7	2.7	1.6	-	-	-	-	-	-	-	-	-
	MAD(z) (cm)	1.8	2.6	2.7	2.1	2.1	1.6	0	-	-	-	-	-	-	-	-	-
CC vs. IC	p-value	0.07	**0.005**	**0.05**	0.86	0.20	0.09	-	-	-	-	-	-	-	-	-	-
Exp. C	Binning x (cm)	0-8	8-16	16-24	24-32	32-40	40-48	48-56	56-64	64-72	72-80	80-88	88-96	96-104	104-112	112-120	120-128
Control condition	Size N	12	14	15	15	15	15	15	15	15	15	15	15	15	15	15	15
	Median(z) (cm)	12.3	9.6	8.1	7.1	6.7	8.5	9.0	9.0	9.5	11.4	11.9	11.4	12.8	11.6	12.7	11.6
	MAD(z) (cm)	0.9	1.2	3.3	4.1	4.2	3.4	3.7	3.8	3.1	3.4	3.5	4.1	4.6	4.2	4.3	3.7
Impover -ished condition	Size N	13	13	11	9	8	7	4	3	1	1	0	0	0	0	0	0
	Median(z) (cm)	11.8	11.1	7.6	10.1	7.8	3.4	5.4	4.0	3.7	1.8	-	-	-	-	-	-
	MAD(z) (cm)	2.1	3.1	2.6	2.5	3.8	3.4	0.4	1.5	0	0	-	-	-	-	-	-
CC vs. IC	p-value	0.72	0.61	0.75	0.63	0.63	**0.02**	**0.006**	**0.002**	-	-	-	-	-	-	-	-
Exp. D	Binning x (cm)	35-43	43-51	51-59	59-67	67-75	75-83	83-91	91-99	99-107	107-115	115-123	123-131	131-139	139-147	147-155	155-163
Control condition	Size N	12	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
	Median(z) (cm)	12.7	10.5	9.4	9.4	7.8	7.2	8.4	9.8	10.6	10.2	12.1	11.4	11.7	13.0	14.5	15
	MAD(z) (cm)	1.5	2.1	2.0	2.1	3.0	2.9	2.8	2.0	2.5	3.1	2.7	3.1	2.7	2.6	2.1	2.0
Impover -ished condition	Size N	9	12	13	13	13	14	14	14	14	13	12	9	8	4	3	1
	Median(z) (cm)	11.6	10.7	9.8	7.2	7.8	9.2	11.1	12.2	11.3	10.5	11.0	12.1	8.2	6.7	3.2	0
	MAD(z) (cm)	2.4	1.8	3.3	3.0	2.5	2.9	4.2	4.3	5.0	4.9	5.3	5.2	5.1	4.0	3.7	0
CC vs. IC	p-value	**0.03**	0.85	0.75	0.61	0.79	0.39	0.26	0.35	0.91	0.51	0.70	0.82	0.12	**0.01**	**0.003**	-

Table S1. Comparison of the altitude distributions in each experiment (Exp. A, Exp. B, Exp. C, and Exp. D) with a binning of 8 cm along the abscissa. For each experiment and both conditions (control condition CC and impoverished condition IC), the size of the sample in the binning (N), the median, and Median Absolute Deviation (MAD) are given. Mann–Whitney U tests are used to compare altitude binning distribution by pairs : control conditions (CC) versus impoverished visual conditions (IC) in each experiment (Exp. A, Exp. B, Exp. C, and Exp. D). Significant probabilities (CC vs. IC) are in bold. All data in figure 1 are in this table.