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The neonicotinoid clothianidin interferes with navigation of the solitary bee *Osmia cornuta* in a laboratory test

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ABSTRACT Pollinating insects provide a vital ecosystem service to crops and wild plants. Exposure to low doses of neonicotinoid insecticides has sub-lethal effects on social pollinators such as bumblebees and honeybees, disturbing their navigation and interfering with their development. Solitary Hymenoptera are also very important ecosystem service providers, but the sub-lethal effects of neonicotinoids have not yet been studied well in those animals. We analyzed the ability of walking *Osmia* to remember a feeding place in a small environment and found that *Osmia* remembers the feeding place well after four days' training. Uptake of field realistic amounts of the neonicotinoid clothianidin (0.76 ng/bee) altered the animals' sensory responses to the visual environment and interfered with the retrieval of navigational memory. We conclude that the neonicotinoid clothianidin compromises visual guidance and the use of navigational memory in the solitary bee *Osmia cornuta*.

KEYWORDS *Clothianidin, neonicotinoid, memory retrieval, Osmia cornuta, learning, navigation, arena experiment*

Pollinating insects contribute significantly to agricultural productivity and solitary bees play a key role in the global pollination market (Garibaldi et al., 2013; Klein et al., 2007). The prevalent use of pesticides in crop protection is suspected of posing a threat to pollinating insects in agricultural landscapes (Carreck, 2015; van der Sluijs et al., 2013). Sub-lethal doses compromise memory and learning, foraging, homing behaviour, colony development and reproductive output (Desneux et al., 2007; Godfray et al., 2014; Kevan and Menzel, 2012). The effects of sub-lethal doses of neonicotinoids on solitary bees have not yet been studied in depth, with only few reports emphasizing a negative impact on, e.g. larval survival rate (Tesoriero et al., 2003), foraging behaviour (Gels et al., 2002; Mommaerts et al., 2010) and species composition at the community level (Blacquière et al., 2012; Brittain et al., 2010).

Here we focus on the effect of clothianidin on navigation of *Osmia cornuta* in a laboratory test. Like social bees, *Osmia* needs to return to the nest to supply their larvae with food, and to select feeding sites in the most efficient manner. Our laboratory test requires walking *Osmia* to locate a feeding site in a small arena by visual cues at the feeding site and extra arena cues, the panorama. We show that the use of the acquired visual memory is compromised by the uptake clothianidin.

RESULTS AND DISCUSSION

Osmia bees are trained to find a fixed feeding site in the arena using a blue local cue and visual patterns at the panorama. Naïve and trained *Osmia* bees explore the arena but have a tendency for walking along the border of the arena. We, therefore, excluded trajectories within areas up to 2 cm along the vertical frame, the border of the arena. Since we use the trajectories as measures of the search behaviour we

excluded resting times, periods of 6 s of not walking. This procedure does not induce a bias in the comparison between naïve control bees and treated bees because resting times did not differ between these two groups. These procedures lead to summed periods of active time in each of the four quadrants as a measure of spatial exploration. Representative examples of walking trajectories are presented in the appendix 1. Naïve control bees did not prefer any of the four quadrants, but trained control bees spent more active time in the quadrant of the local cue, indicating that they recognized and learned the local cue as the rewarding site (Fig. 1A, B).

In the dissociation test (see Methods), the trained control bees walked from the local cue to the panorama location more frequently than naïve control bees (Fig. 2), indicating that they also remembered the panorama-related location, although the local cue overshadowed the selection of the panorama location.

Naïve pesticide-treated bees spent significantly more time in quadrants 1 and 2 than naive control bees (appendix 2A), indicating that they responded differently to cue and/or panorama. We next asked if the locomotor behaviour changed in treated animals and found no difference in walking speed between control and treated animals, but in the straightness of their walks (appendix 3). This difference, however, does not cause a preference of treated animals for the quadrants 1 and 2. Most importantly, trained treated animals did not distribute their active times equally in the four quadrants (Fig. 3). Particularly the quadrant with the local cue (Q2) was not preferred over the other quadrants, neither in naïve nor in trained animals. This result shows that clothianidin interferes with the retrieval of the memory for the learned guiding features in the arena, the local cue and the panorama. Thus clothianidin treatment leads to a block of memory retrieval for cues guiding *Osmia* to the location of the feeding site, the local cue and the panorama.

Laboratory tests of spatial learning in animals have a long tradition in behavioural biology (Jacobs and Menzel, 2014; Tolman, 1948; Wiener et al., 2011). The test conditions in our experiments exposed the animal to a local cue and to extra-maze cues that either in addition or alone signalled a location to the animal. *Osmia* like bumblebees in a similar test situation learned both the cue and the panorama-related location (Jin et al., 2014). This rather simple training and test procedure allows studying basic forms of navigation under strictly controlled conditions. *Osmia* is compromised in its ability to use this basic form of navigational memory after exposure to a neonicotinoid. Neonicotinoids act as agonists to nicotinic acetylcholine receptors (nAChR) in the insect brain leading to lasting over excitation followed by a block of synaptic transmission (Matsuda et al., 2001). nAChR are particularly frequent in central sensory projections and higher order interneurons, e.g. in the mushroom body. It is thus likely that high order sensory integration, learning, memory formation and memory retrieval may be affected by neonicotinoids.

Navigation of honeybees under natural conditions was found to be disturbed after exposure to sub-lethal doses of different neonicotinoids (Henry and Decourtye, 2013) although their sensory and motor performance as well as their ability to use the sun compass for navigation was not affected (Fischer et al., 2014). In contrast, naïve clothianidin-treated *Osmia* spent more time in a sub-region of the arena close to the tilted black stripes of the panorama and furthest away from the blue board of the local cue, indicating a possible visuomotor effect of clothianidin on spontaneous *Osmia* behaviour. Because motor performance did not change we interpreted the clothianidin effect on naive *Osmia* as a modulation of visual perception that was not seen in the honeybee possibly because of less well-controlled conditions.

Our study documents two major differences between the control group and the treated group. First, naïve treated animals and trained treated animals do not distribute their search trajectories evenly across the arena but preferred a sub-region not including the blue cardboard as a local cue (Appendix 2). Second, trained treated animals were unable to use the memory they had acquired during training. The first effect indicates that clothianidin treatment alters the sensory response to environmental cues arising from extra-maze signals. This effect is particularly important because the panorama-related cues allow the animal to localize the feeding place without the local cue. Although we cannot yet identify particular panorama signals becoming either more or less attractive following clothianidin treatment, it is obvious that uptake of this neonicotinoid alters the animals' detection and/or evaluation of visual signals. The second effect documents that the clothianidin-treated animals were not able to find the learned quadrant with the feeding site.

The clothianidin dose used in our study (0.76ng/animal) lies within the range of expected uptake from treated oilseed crops. Residue intake has been estimated to range from 4.27 to 13.65 ng/bee/day (Authority, 2013). Similar doses during chronic exposure to a different neonicotinoid (imidacloprid) were used by Tasei et al. (Tasei et al., 2000), who found in bumblebees reduced survival of workers and less brood production. Rundlöf et al. (2015) found 6.7 - 16 ng/ml Clothianidin in the nectar store of honeybees foraging in oil rape fields that grow from seeds coated with Clothianidin. The respective values for bumble bees are 1.4-14 ng/ml. One can estimate that a honeybee or a bumble bee will usually collect about 50 µl of nectar on one foraging trip. Thus each honeybee takes up about 0.335-0.8 ng Clothianidin during one foraging trip (bumblebee 0.07 – 0.7 ng). Depending on foraging time and distance travelled in consecutive foraging trips a considerable amount of the collected

Clothianidin will be taken up by the body of the bee. We fed each Osmia bee 0.76 ng, a dose of Clothianidin rather close to what can be expected from these data.

These results document that clothianidin, taken up at a concentration lower than 1ng/bee, blocks the retrieval of memory necessary for navigating towards a learned location, corroborating findings from previous studies on solitary bees (Abbott et al., 2008) and social bees (Fischer et al., 2014; Schneider et al., 2012). Further tests are needed to determine the dose-dependent effects of neonicotinoids on learning, memory formation and memory retrieval in the context of navigation in order to evaluate the sensitivity of solitary bee species to neonicotinoid uptake.

MATERIAL AND METHODS

Animals: Osmia cornuta emerged from the pupae during early spring (March to May). The pupae were purchased from M. Herrmann, WAB – Mauerbienenzucht, Sonnentauweg 47, D - 78467 Konstanz, and stored in the refrigerator at 4°C until 1 day before use. Virgin females were trained 1 day after emerging from pupation. We cut the right wing of each bee in order to prevent her from flying. Four groups of animals were tested: naïve bees without exposure to clothianidin (n=20, naïve control); trained bees without exposure to clothianidin (n=12, trained control); naïve animals treated with clothianidin (n=10, naïve treated), and trained bees treated with clothianidin (n=10, trained treated).

Experimental setup, training and tests: The animals were trained and tested in an arena (Fig. 4) and video recorded (15 fps) using a web camera inserted through a hole in the centre of the ceiling of the arena. Exploratory behaviour can be stimulated with evenly distributed odour in the air space above the arena. We placed a piece of paper

soaked with 50 l liquid citral at the centre of the ceiling. All structures inside the arena were assembled symmetrically, and therefore no geometrical cues were available to the bees other than the panorama (simple patterns on inner walls, see Fig.4). The animals were trained during an exploratory period during which it fed three times diluted honey (1:1 honey/water based on volume) on a 5 cm x 5 cm blue cardboard paper (local cue). Training time may be different for different animals (2-15 minutes) depending on how long it took them to find the reward. Training sessions of each animal were performed at the same time of day during four consecutive days. Notice that the odour of the honey does not interfere with the test procedure (see below). On the fifth day, the trained bees (control or treated) were tested in one of the following two ways: 1. Both local cue and panorama were rotated 90°clockwise (Fig. 4C, the direction of 90° rotation was randomly chosen); 2. The local cue was rotated 180° while the panorama was rotated 90°clockwise (Fig. 4D). In both tests, the honey water was removed, and the ground cardboard was gently moved over to the test ground without moving the plastic frame. In this way, any putative odour cues were removed, and the search behaviour can be guided only by visual cues. The starting place is randomly chosen from any quadrant where the local cue is not located. Each test lasted for 15 minutes. Naïve animals (control and treated) were not trained and were tested on the day they emerged.

Pesticides preparation: the Clothianidin standard (Sigma Aldrich, Hamburg Germany) was diluted in acetone, mixed with tap water leading to a stock solution (S.S.) of a concentration of 135.77 ppm, and further diluted in two steps to obtain the final concentration in the feeding solution (F.S.) of 0.076 ppm (resp. 0,076 ng/µl). The concentrations of S.S. and the first step of dilution of F.S. were checked with LC-MS/MS (see appendix 4).

Clothianidin treatment: Preliminary experiments showed that administration of 1.25 ng clothianidin per animal leads to a mortality rate of 70% within 2 hours after intake. We, therefore, reduced the concentration to 0.76 ng/animal. Furthermore, we observed that naïve animals behaved normally in the arena 60 minutes after uptake of 10µl of the clothianidin in diluted honey solution (see appendix 3A,B, "control" columns). In the tests of treated naive animals, the animals were fed with 0.76 ng/bee clothianidin one hour after they were caught and then incubated for 1 hour before the test. In the retention test after 4 days of training, *Osmia* was fed with 0.76 ng/bee clothianidin immediately after the last training and 1 hour before the test on the 5th day. All treated animals were kept in a dark wooden box after uptake of the pesticide for pesticide incubation.

A total of 22 *Osmia* bees were trained, 12 control bees were tested on the 5th day and 10 treated bees after imbibing 10µl of a mixture of honey-water solution and pesticide. In order to quantify the learning effect, 30 naïve bees (20 controls, 10 treated) were tested without training.

Statistical analyses: The normality of the distribution of the distance travelled was evaluated by the Shapiro test. The differences were tested by a one-way ANOVA. The trajectories of walking in the tests were analysed with respect to the active times spent in one of four quadrants (Fig. 1 and 3). The summed active times spent in each quadrant was calculated during the first four minutes of active time. Since the values were not normally distributed (Shapiro test, p-values <0.01) the Friedman rank sum test was applied. All statistical analyses were performed with RStudio (0.98.507) for R version 3.0.2. The Friedman test for repeated measurement was provided by the

package muStat (Wittkowski and Song, 2007). Setout directions were analysed with the independent t test (Fig. 2).

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References

Abbott, V. A., Nadeau, J. L., Higo, H. A. and Winston, M. L. (2008). Lethal and sublethal effects of imidacloprid on Osmia lignaria and clothianidin on Megachile rotundata (Hymenoptera: Megachilidae). *J Econ Entomol* **101**, 784–796.

Authority, E. F. S. (2013). Conclusion on the peer review of the pesticide risk assessment for bees for the active substance imidacloprid. *EFSA J.* **11**, 1–55.

Blacquière, T., Smagghe, G., van Gestel, C. a M. and Mommaerts, V. (2012). Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**, 973–92.

Brittain, C. A., Vighi, M., Bommarco, R., Settele, J. and Potts, S. G. (2010). Impacts of a pesticide on pollinator species richness at different spatial scales. *Basic Appl. Ecol.* **11**, 106–115.

Carreck, N. (2015). Are We Beginning To Understand Worldwide Colony Losses? *Bee World* **91**, 20–21.

Desneux, N., Decourtye, A. and Delpuech, J. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* **52**, 81–106.

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B. and Menzel, R. (2014). Neonicotinoids interfere with specific components of navigation in honeybees. *PLoS One* **9**, e91364.

Garibaldi, L., Steffan-Dewenter, I. and Winfree, R. (2013). Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science (80-.).* **339**, 1608–1611.

Gels, J. A., Held, D. W. and Potter, D. A. (2002). Hazards of insecticides to the bumble bees Bombus impatiens (Hymenoptera: Apidae) foraging on flowering white clover in turf. *J. Econ. Entomol.* **95**, 722–728.

Godfray, H. C. J., Blacquiere, T., Field, L. M., Hails, R. S., Petrokofsky, G., Potts, S. G., Raine, N. E., Vanbergen, A. J. and McLean, A. R. (2014). A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc. R. Soc. B-Biological Sci.* **281**,.

Henry, M. and Decourtye, A. (2013). Ecological relevance in honeybee pesticide risk assessment: developing context-dependent scenarios to manage uncertainty. *Front. Physiol.* **d**, 1–3.

Jacobs, L. F. and Menzel, R. (2014). Navigation outside of the box: what the lab can learn from the field and what the field can learn from the lab. *Mov. Ecol.* **2**, 3.

Jin, N., Landgraf, T., Klein, S. and Menzel, R. (2014). Walking bumblebees memorize panorama and local cues in a laboratory test of navigation. *Anim. Behav.* **97**, 13–23.

Kevan, P. G. and Menzel, R. (2012). The plight of pollination and the interface of neurobiology, ecology and food security. *Environmentalist* **32**, 300–310.

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C. and Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proc. Biol. Sci.* **274**, 303–313.

Matsuda, K., Buckingham, S. D., Kleier, D., Rauh, J. J., Grauso, M. and Sattelle, D. B. (2001). Neonicotinoids: Insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* **22**, 573–580.

Mommaerts, V., Reynders, S., Boulet, J., Besard, L., Sterk, G. and Smagghe, G. (2010). Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. *Ecotoxicology* **19**, 207–215.

Rundlöf, M., Andersson, G. K. S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B. K., Pedersen, T. R., Yourstone, J., et al. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature* 521, 77–80.

Schneider, C. W., Tautz, J., Grünewald, B. and Fuchs, S. (2012). RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of Apis mellifera. *PLoS One* **7**, e30023.

Tasei, J. N., Lerin, J. and Ripault, G. (2000). Sub-lethal effects of imidacloprid on bumblebees, Bombus terrestris (Hymenoptera : Apidae), during a laboratory feeding test. *Pest Manag. Sci.* **56**, 784–788.

Tesoriero, D., Maccagnani, B., Santi, F. and Celli, G. (2003). Toxicity of three pesticides on larval instars of Osmia cornuta : preliminary results. *Bull. Insectology* **56**, 169–171.

Tolman, E. C. (1948). Cognitive Maps in Rats and Men. *Psychol. Rev.* **55**, 189–208.

Van der Sluijs, J. P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J.-M. and Belzunces, L. P. (2013). Neonicotinoids, bee disorders and the sustainability of pollinator services. *Curr. Opin. Environ. Sustain.* **5**, 293–305.

Wiener, J., Shettleworth, S., Bingman, V. P., Cheng, K., Healy, S., Jacobs, L. F., Jeffery, K. J., Menzel, R., Mallot, H. A. and Newcombe, N. S. (2011). Animal navigation--A synthesis. In *Animal thinking*, p. 1:33. MIT Press.

Wittkowski, K. and Song, T. (2007). Package " muStat ."

Figures

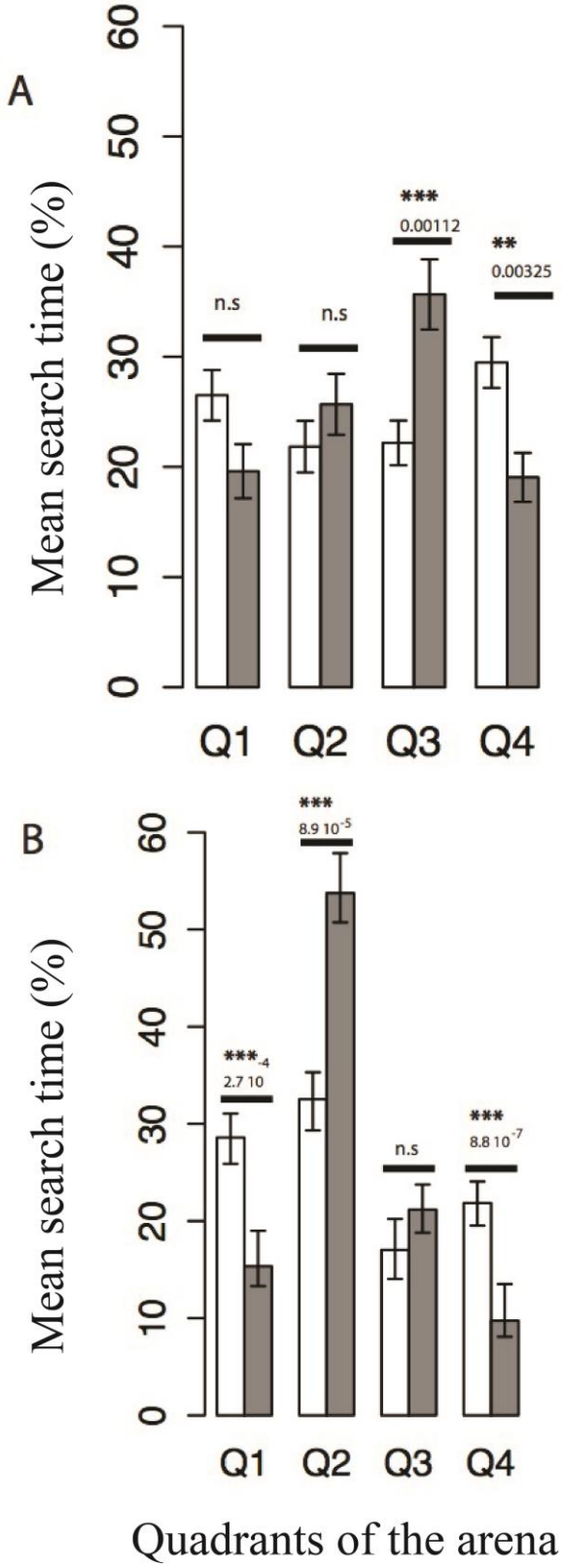

Fig. 1. Distribution of summed and normalized active times within the four quadrants (Q1 – Q4) of the arena during tests of control bees before (open columns) and after training (dark columns). The feeding site on the local cue was located in Q2. (A) Panorama and local cue are co-localized. (B) Panorama and local cue are dissociated by a rotation of 90° relative to each other during the testing. The ordinate gives the mean percentage of summed and normalized active time. The Friedman test for repeated measurements was used to test the learning effect of training to the feeding site (NS: not significant). Naïve control group: chi-square = 7.59, p-value = 0.055, trained control group: chi- square = 19.14, p-value < 0.001, naïve treated group: chi-square = 39.27, p- value < 0.001, trained treated group: chi-square = 79.59, p-value < 0.001.

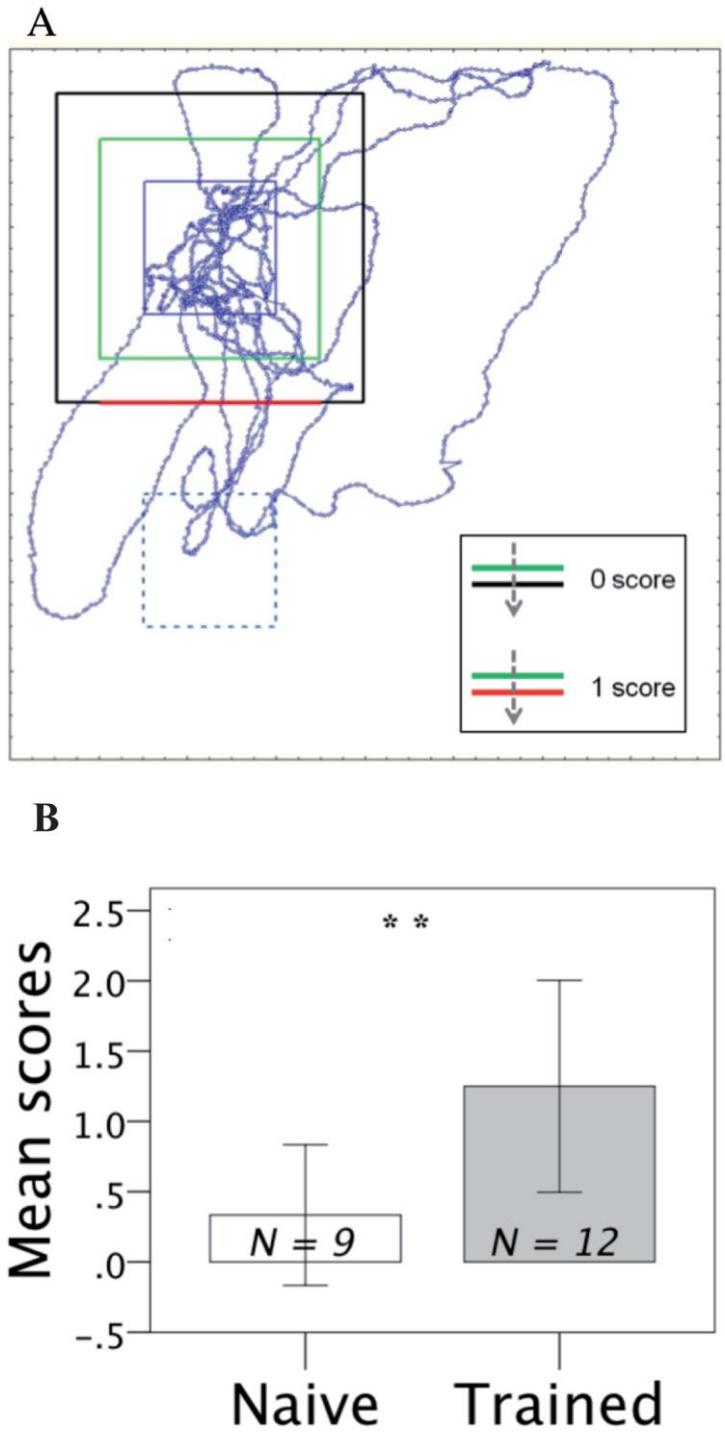

Fig. 2. Directional components of outbound trajectories from the local cue during the dissociation test in control bees. (A) Local cue and panorama locations are dissociated by 90°. The lines around the local cue (blue solid line square) define an inner box

(green, length of sides 4 cm) and an outer box (black and red lines, length of sides: 13.8 cm; the length proportion of red: black is 5:23). The blue dotted line box marks the feeding place indicated by the panorama. Trajectories crossing first the green lines and then the black or red lines were counted as departing from the local cue. Those crossing the red line were given a score of 1 because they indicate a departure from the local cue area towards the panorama location. Trajectories crossing the black lines scored 0. Only the first and second departures of each bee were assessed. Thus the score of a single bee could be 0, 1 or 2. (B) Mean departure scores for the dissociation of 90°. Since animals from the naïve and the trained groups are different the two groups are independent from each other. Therefore, we applied an independent samples t-test with the null hypotheses that the panorama is not guiding the trained animals to the feeding site, and therefore the probabilities for naïve and trained animals to move from the local cue to the panorama related location are the same. The null hypothesis can be rejected (**$P < 0.01$, error bars give SD). The number of animals tested is given in the columns.

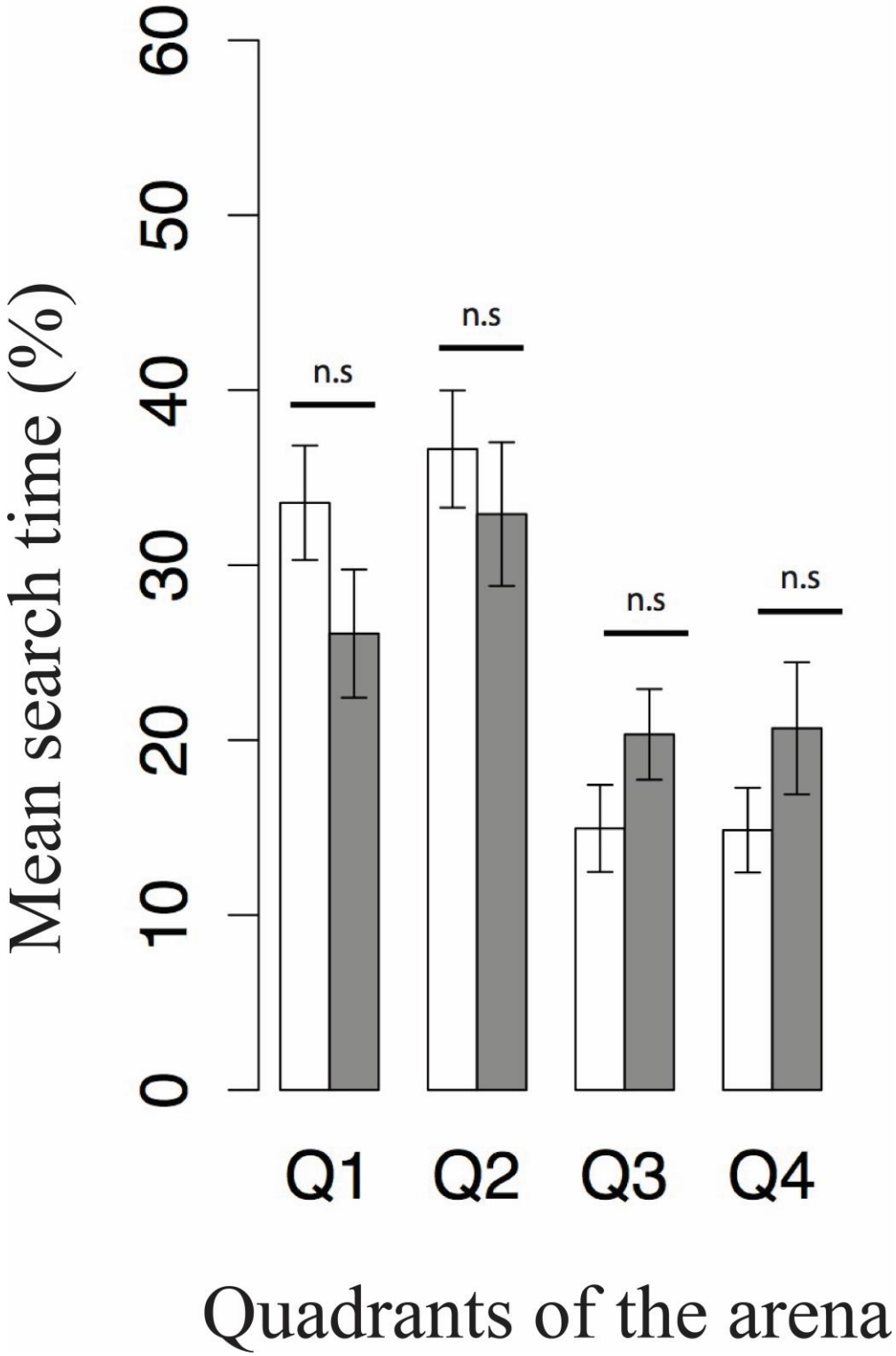

Fig. 3. Distribution of cumulative active time in treated bees. Open columns give the summed active time for naive treated animals, and dark columns that for trained treated animals. The Friedman test for repeated measurements was used to test whether the null hypothesis (no difference in the active times before and after training) applies (NS: not significant). The Friedman test for repeated measurements (with minutes as repeated measurements) was used to test the difference in each quadrant for a particular group. Naïve control group: chi-square = 7.59, p-value = 0.055, trained control group: chi- square = 19.14, p-value < 0.001, naïve treated group: chi-square = 39.27, p- value < 0.001, trained treated group: chi-square = 79.59, p-value < 0.001.

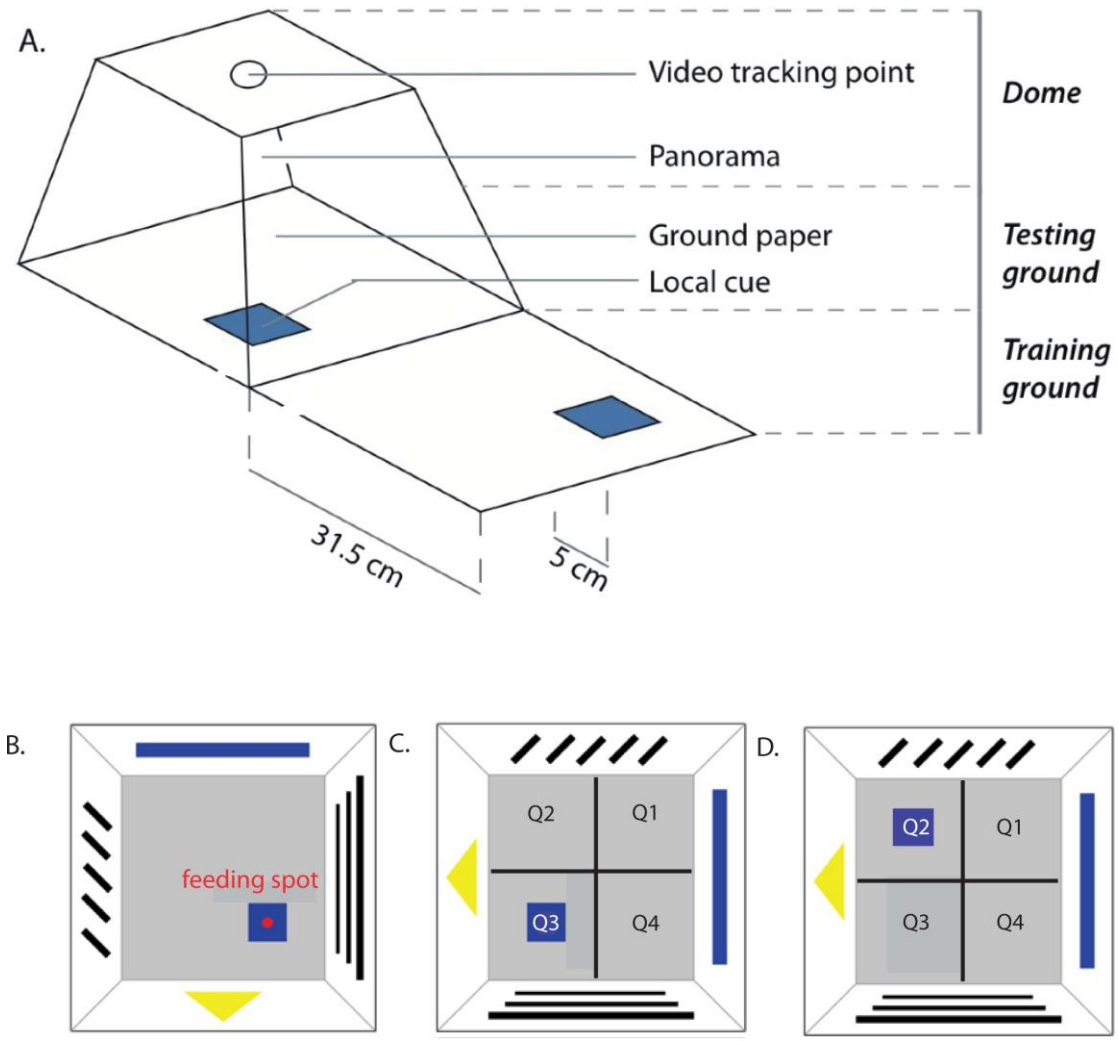

Fig. 4. Experimental arrangement. (A) The ground of the arena consisted of a plastic board on which a grey cardboard paper could be moved across the ground. A transparent plastic frame (31.5 cm x 31.5 cm x 10 cm) confined the range of the arena ground. The local cue (blue board of 5 x 5 cm) marked the reward in its centre. The walking tracks of the animals were recorded by a camera on the top of the dome. (B) Bird's eye view of the arena (local cue on the ground and panorama patterns on walls) during training. The red spot indicates the location of the feeding spot, the opening of a transparent plastic tube. (C) and (D) Orientation of the ground paper and the

panorama for the tests. In (C), the ground paper and the panorama were rotated by 90°. In (D), the local cue was rotated 180° while panorama was rotated 90°clockwise (dissociation test). The feeding capillary was removed. These figures also show the division of the arena into 4 quadrants (Q1 to Q4) for the purpose of the statistical analyses.