Methods supplement to:
Very rapid multi-odour discrimination learning in the ant *Lasius niger*

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Contents

Study species and maintenance

We used 8 queenless colony fragments (c. 1000 workers) of the black garden ant, *Lasius niger* (Linnaeus), collected from 8 different colonies on the University of Regensburg campus. Colonies were housed in a plastic box (40×30×20 cm) with a layer of plaster on the bottom. Each box contained a circular plaster nest (14 cm diameter, 2 cm high). Colonies contained c. 1000 workers and small amounts of male brood. The ants were maintained at room temperature (21-24C°) in a 12:12 light:dark cycle, and fed 0.5M sucrose *ad libitum,* supplemented with *Drosophila melanogaster*. Colonies were deprived of food for four days prior to each trial. Water was provided *ad libitum*.

Materials and setup

Innate odour preference tests (pretraining controls)

Innate preference for three of the odour pairs used was tested. This was done by allowing groups (5-20) of naïve ants onto a Y-maze (arms 10cm long, 1cm wide, tapering to 2mm at the bifurcation) via a drawbridge. The arms and stem were covered in disposable paper overlays. Arm overlays were scented with one of the odours used, by keeping them in a sealed box containing 5 drops of the odour (rose, orange, blackberry or lemon food flavouring, Seeger, Springe, Germany) on a glass petridish for at least two hours. As ants passed a line 2cm away from the end of each arm, they were gently brushed into a holding container below the arm and excluded from the rest of the test, to prevent pseudoreplication. After 5-20 ants had been tested, the maze was cleaned, the overlays replaced, and the odour sides were switched, to control for potential side bias. This was done four times, until 20-60 ants were tested from each colony for each odour pair. 4-6 colonies were tested for each odour pair. We tested the odour pairs rose vs. orange (exact binomial test, no preference, 128 / 240 chose orange, 54%, P = 0.33), blackberry vs. orange (no preference, 81 / 180 chose orange,

45%, P = 0.21), and rose vs. blackberry (weak aversion to rose, 378 / 820, 46%, P = 0.028). Lemon was also used in the 4-odour treatment (see below), but pairwise preference tests against this odour were not carried out, as it was only ever used as either the worst or best odour in a 4-odour set.

A US control (in which the odours are provided without reward) was not possible, as without reward ants would refuse to return to the apparatus over multiple visits. We chose to forgo a CS control (exposing ants to various sucrose solutions without odours), as our experiment was blocked so as to exclude non-learning explanations for a deviation from random choice.

General procedure

We carried out four experiments, all using similar methods. First, ants are trained on a linear runway to associate odours with rewards, and then binary preference between the trained odours is tested on a Y-maze.

Training

An experiment begins by connecting a colony to a linear runway (20cm long, 1cm wide) covered in a replaceable scented paper overlay. A drop of sucrose is placed at the end of the runway, flavoured similarly to the runway (1µl food flavouring per ml sucrose solution). The first ant to contact the drop and begin drinking is marked with a dot of acrylic paint on the abdomen. All other ants are returned to the nest and further access prevented. The marked ant is allowed to drink to satiation and return to the nest. While in the nest, the runway is replaced by a new runway covered by a fresh, differently scented paper overlay. A drop of matching sucrose with a different molarity is placed at the end. Once the painted ant has finished unloading, she is allowed back onto the runway, allowed to drink again, and allowed to return. The training procedure is repeated a variable number of times and to a variable number of sucrose/odour combinations, depending on the experiment. Pheromone deposition behaviour was recorded for all return journeys to and from the food, but not analysed (see S2). We invite other researchers to explore these data and use them in any way they like.

Testing

After training, the linear runway is replaced by a Y-maze as described for the innate odour preference test. The painted ant is allowed onto the Y-maze, each arm of which is scented with a different odour, and the initial choice (crossing a line 2cm from the bifurcation) and the final choice (crossing a line 2cm from the arm end) is noted. Only the final decisions are analysed. A piece of paper is laid at the end of the chosen runway arm, allowing the ant to run onto it, whereupon it is moved back to the drawbridge. The ant invariably continues running outwards towards the Y-maze. The Y-maze is replaced with a different pre-prepared Y-maze, with a different odour pair on the arms. The ant is allowed to make a second choice, and the process is repeated a third time, with a third pair of odours. After testing, the ant is permanently removed from the colony.

Experimental details, blocking procedures, and tested odour pairs

Four experiments were carried out in total: two odours – four exposures per odour, three odours – three exposures, four odours – two exposures, and four odours – one exposure (see table 1).

We attempted to block for as many variables as possible in each experiment. However, complete blocking for all variables was not possible, as it would require a prohibitively large sample size. Similarly, we could not test all pairwise comparisons in experiments 3 & 4 (4 odours).

In experiment 1 a familiarisation visit was carried out before the main training visits, in which the runway was unscented and offered 0.5M unscented sucrose at the end. We chose not to carry out familiarisation visits in the other experiments, as they resulted in negative incentive contrast effects (Wendt et al. 2019), and a refusal to consume the lower quality foods (see below). We fully blocked

for the odour of the two molarities (half the ants received 1.5M sucrose associated with rose, the other half with blackberry), which molarity was present on the first training visit, and which molarity-associated odour was on the left of the Y-maze during testing.

In experiment 2, half the ants had rose associated with 1.8M and blackberry with 0.2M, and the other the opposite; orange was always associated with 0.6M. Training order was always fixed in ascending order of quality (0.2, 0.6, 1.8, 0.2, 0.6, 1.8, 0.2, 0.6, 1.8). This was done to ensure full drinking of, and exposure to, even the lowest molarities: ants experience strong negative incentive contrasts (Wendt et al. 2019), and will often refuse lower quality food if they have recently experienced a higher quality. Moreover, *L. niger* ants show a very strong primacy effect (Oberhauser et al., in prep.), meaning that all else being equal, they strongly prefer the first association they make. Thus, our design biases the ants towards choosing the worst odour, making any result showing a choice of the best (and least-primal) odour more convincing. All three odour/molarity combinations pairs were tested. We either tested 0.2M vs. 0.6M first, followed by 0.6M vs 1.8M, or the reverse. We chose to test 0.2M vs 1.8M last, as this is objectively the easiest task, and would be more resistant to any distress caused by repeated handling of the ant. The location of the high-quality odour was alternated on each trial (L,R,L or R,L,R, also blocked along with the variables above).

In experiments 3 and 4, we could not test all possible odour/quality binary choices. We thus chose to test all neighbouring pairs, that is 0.2M vs 0.4M, 0.4M vs 0.8M, and 0.8M vs 1.6M. We chose to always test 0.4M vs 0.8M first, as we considered this the most critical test. This is because success in this test absolutely requires an explicit association between the two odours and their associated molarities (see discussion). We chose to test 0.2M vs 0.4M second, as these molarities were the both the lowest, and the first encountered, which may result in weaker learning. The location of the high-quality odour was alternated on each trial, as in experiment 2.

Statistical analysis

The entire statistical analysis workflow, with code and output, is provided in S2. The entire dataset is provided in S3.

Data were analysed in R V.3.6.1 (R Core Team 2018) via RStudio (RStudio Team 2015). Data from experiment 1 were directly analysed with exact binomial tests, as low sample size and very clear results precluded more advanced analysis. In experiments 2-4, we first tested whether colony-level effects or side biases influenced our data. To do this, we constructed mixed-effect models (Bates et al. 2014) predicting ant decision (correct/incorrect) by whether the correct side was left or right, in interaction with the pairwise choice the ant was making (e.g. 0.2M vs 0.4M), with colony as a random effect. We found no evidence for colony-level random effects (see S2), but did find a side bias (see results). Nonetheless, since our experiment was well-blocked for side, we chose to analyse the data for each pairwise choice using exact binomial tests, against a null hypothesis of 0.5. We felt that the power and simplicity of these tests outweighed their inability to incorporate the side-bias effects.

References cited in the supplement

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