Serial reversal learning in nectar-feeding bats

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Introduction

Methods

Study site and subjects

The experiment was done in June and July 2017 at La Selva Biological Field Station, Province Heredia, Costa Rica. Male and female individuals of the species *Glossophaga commissarisi*, were captured from the wild for the experiment. The bats were attracted to a particular location in the forest using sugar-water (see **Reward** below) as bait and then caught in mist-nets. The bats were sexed and the selected individuals were were then taken to another location in the rainforest where two flight-cages (4 x 6 m) were set up. The flight-cages had mesh walls and therefore the same climactic conditions as the surrounding environment. A group of four bats at a time were put into a flight cage. All the individuals in a group were the same sex. The bats were weighed and radio frequency identification (RFID) tags that were uniquely assigned to each bat were placed around their necks as collars. The bats were then released into the flight-cages so they could fly within them freely.

Before the start of the experiment the procedure was tested with four females and refinements were made to the procedure. The data from these individuals were not analyzed. 16 bats participated in the main experiment. At the end of the experiment, the RFID collars were removed and the bats were weighed to make sure they had not lost too much weight (see **Supplementary Information - Table 1** for details). No blinding was done as all the data collection was completely automatized. Two of the bats did not drink sufficient nectar and were released before the end of the experiment and not replaced. The data from these two individuals were not analyzed. Thus, 14 bats in total (7 males and 7 females) completed the experiment and the data from these animals were analyzed.

Animal experimental procedures were reviewed and permission for animal experimentation and RFID-tagging was granted by Sistema Nacional de Areas de Conservación (SINAC) at the Ministerio de Ambiente y Energía (MINAE), Costa Rica.

Experimental Setup

Reward

The reward received by the bats during the experiment was also their main source of food. The reward was a 17% by weight solution of sugar dissolved in water (prepared fresh everyday), hereafter referred to as 'nectar.' The sugar consisted of a 1:1:1 mass-mixture of sucrose, fructose and dextrose. The nectar was thus similar in composition and concentration to the nectar produced by wild chiropterophilous plants (Baker, Baker, and Hodges 1998).

Flower and pump setup

Each flight cage had a square plastic frame in the center (2x2x1.5m). Eight reward-dispensing devices - hereafter referred to as 'flowers' - were fixed in a radial pattern on this frame, two on each side of the square (see Figure 1) with a minimum distance of 40 cm between adjacent flowers. This is a distance discriminable by these bats (Thiele and Winter 2005). Each flower had the following parts: an RFID reader mounted on a plastic cylinder around the head of the flower; an infra-red light-barrier beam; an electronic pinch valve through which a PVC tube was placed and fixed to the head of the flower

A single stepper-motor pump was placed in the center of the plastic frame. The pump contained a 25 mL Hamilton glass syringe (Sigma Aldrich) which was connected to the tubing system of the flowers through 5 pinch valves. These pinch valves controlled the flow of liquid from the pump to the system and from a reservoir of liquid to the pump. This reservoir (500 mL thread bottle, Roth, Germany) was filled with fresh nectar everyday and connected to the syringe through the valves. The nectar was then pushed by the syringe through the valves into the tubes.

When an RFID-tagged bat approached a flower, the individual RFID number was read by the reader. If the bat then poked its nose into the flower and broke the light barrier, it triggered the release of a reward. The pinch valve opened and the pump moved the correct number of pre-programmed steps to dispense nectar to the head of the flower. The bat could easily hover in front of the flower and lick this up. Only when both events occurred, i.e., the RFID reader detected a bat and the light-barrier was broken, would a reward be triggered.

The flowers and the pump were connected to a Lenovo ThinkPad laptop computer, which ran the experimental programs and the programs used to clean and fill the systems: PhenoSoft Control 16, PhenoSoft GmBH, Berlin, Germany. The raw data were also recorded to this computer as comma-separated values (CSV) files.

Experimental procedure

Every day at around 1000 h the old nectar was emptied from the system. The system was rinsed and filled with plain water until 1500 h , when it was filled again with fresh nectar. Twice a week the system was filled with 70% ethanol for an hour to prevent microbial growth, then repeatedly rinsed with water.

4 bats were placed in a flight-cage in a group, and all the bats were the same sex. There were 4 such groups in total, and data were collected simultaneously from two groups, one in each flight-cage. Each bat was uniquely assigned 2 adjacent flowers on the same side of the square frame, out of the array of 8. These flowers were programmed to reward only 1 of the 4 bats in the cage. After the system was filled with fresh nectar at approximately 1700 h, the program was left running till the next morning for data-collection. Thus, the bats could begin visiting the flowers to collect a reward whenever they chose, which was at approximately 1800 h every night.

During the course of the night, when the syringe of the pump had been emptied, the pump re-filled automatically. This process took 3.75 minutes (SD = ±2.23) in Cage 1, occurring on average `fill_cage1$mean_fills` times (SD = ± `fill_cage1$sd_fills`); and 2.31 minutes (SD = ±0.93) in Cage 2, occurring on average `fill_cage2$mean_fills` times (SD ± `fill_cage2$sd_fills`). About 1 % (SD = ±0.74) of all visits made by the bats over all 3 experimental nights happened during the pump refill events, and the bats did not receive any reward on these visits, even if they were made to the rewarding flower.

Every night the bats were also given ad-libitum supplemental food: 3.5g of hummingbird food (NektarPlus, Nekton) in 100 mL of water and 3.5g of milk powder (Nido 1+, Nestle) in 100 mL of water. They were also given a small bowl of locally-sourced bee pollen.

Experimental design

The experiment proceeded through the following stages.

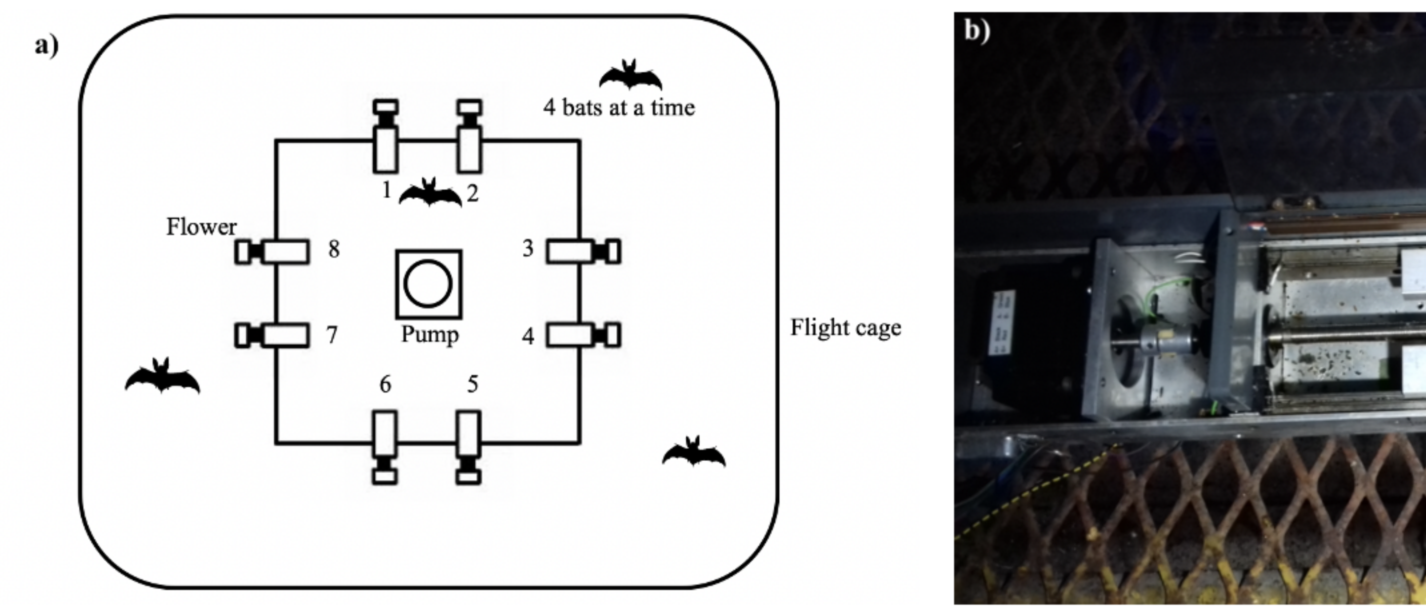


Figure 1: Figure 1: a) Schematic of the cage and flower set-up b) Pump from Cage 1 c) Pump from Cage 2 (#fig:Figure of the setup)

Ad-libitum reward

On the night the naive bats were captured and placed into the flight cages they received ad-libitum reward from all the flowers all night long. To enable the bats to find the flowers a small cotton pad was placed on the flowers, soaked with di-methyl di-sulphide. This is a chemical attractant produced by many bat-pollinated flowers (Helversen, Winkler, and Bestmann 2000). A small drop of honey was applied to the inside of the flowers to encourage the bats to place their heads inside, break the light-barrier and trigger a nectar reward. By the end of the night all the bats had found the flowers and learned to trigger rewards quickly.

Flower training

After the bats had learned to trigger rewards, the next stage of training involved assigning the bats uniquely to two out of the eight flowers in the array. This stage was similar to the *ad-libitum* stage, except the bats could only trigger a reward at their assigned flowers.

Alternation

To ensure that the bats were familiar with both flowers assigned to them they went through one final stage of training: forced alternation between the two assigned flowers all night long.

Main Experiment

In this serial reversal learning task the bats had to choose between a flower that gave 40 μL of nectar and one that gave no reward at all. The location of the rewarding flower was not cued, but through the Alternation phase of training each bat knew the locations of both flowers that were potentially rewarding to it. After a bat had made 50 visits in total to the two flowers a reversal occurred: the previously rewarding flower became the non-rewarding flower and *vice versa*. The batch of 50 visits that occurred between two consecutive reversals (when the locations of the rewarding and unrewarding flowers) was termed a 'reversal block,' including the first 50 visits of a night when the bats had not experienced any reversal at all that night. This occurred at regular intervals of 50 visits until the bat either stopped making visits or reached a maximum of 300 visits in a night. After the bat had made 300 rewarded visits it could no longer receive a reward that experimental night. There were five reversals per night. This stage of the experiment was repeated for three nights in a row. The same flower was the first to be rewarding at the start of every night. Thus, because there were five reversals every night (so six blocks of 50 visits), the last flower to be rewarding on a night was non-rewarding at the start of the next night.

Statistical analysis

All the visits made by the bats during a night, up to a maximum of 300 were included in the analyses. There were 3 experimental nights, divided into six blocks of 50 visits each. At the end of the first five blocks a reversal occurred and the end of the last block was the end of data-collection for the night. Each block was further divided into five bins, each consisting of 10 visits, in order to further examine the bats' behaviour within each block.

We defined a perseverative visit as a visit to the previously-rewarding option just after the occurrence of a reversal, until the first visit to the newly-rewarding option. A generalized linear mixed-model was used to investigate the effect of experimental night and reversal block on the number of perseverative visits. This model was fit in a Bayesian framework using Hamiltonian Monte Carlo in the R package `brms` (Bürkner 2017), which is a front-end for `rstan` (Stan Development Team, 2020). A negative-binomial likelihood function was used for this model. Experimental night, reversal block and their interaction were fixed effects and random slopes and intercepts were used to fit regression lines for each individual animal.

After examining the results of the above analysis, more exploratory analyses were done to examine the bats' choice behaviour from another perspective. Specifically we examined the proportion of visits made to the rewarding flower. This was defined as ratio of the number of visits to the rewarding flower divided by the total number of visits in a bin. The model was fit using a binomial likelihood function, with experimental night, block, bin and their interactions as fixed effects; random slopes and intercepts were used to fit regression lines for the individuals. After examining the results of this model, a second model was fit to these data to take into account the fact that the first night and the first block of each night were qualitatively different from the others. On the first night the animals had had no prior experience of any reversals, and during the first block of every night they had not experienced any reversals on that night, and this was reflected in the fit of the posterior predictions made from the first model. The second model was identical to the first except for the addition of experimental night and block as factor variables, with the first night and the first block of every night as one level and the other nights and other blocks of each night as the other level. The two models were compared using leave-one-out cross-validation, implemented in `brms` using the package `loo` (Vehtari, Gelman, and Gabry 2017).

Weakly informative priors were used. The random intercepts and slopes were given a Normal distribution with a mean of 0, and a standard deviation drawn from a Cauchy distribution with a mean of 0 and a standard deviation of 1. All the models were estimated using 4 chains with a thinning interval of 3, with 1200 warm-up samples and 1300 post-warm-up samples for the model with the first experimental night and block additionally treated differently, and 1000 warm-up samples and 1000 post-warm-up samples for the others.

Visual inspection of the trace plots, the number of effective samples, the Gelman-Rubin convergence diagnostic ($\hat{R}$) and the calculation of posterior predictions for the same clusters were all used to assess the fit of the models. In all of the models the $\hat{R}$ was equal to 1 for all the chains.

The data from all 14 bats that participated in the three experimental nights were included in these models, even though some individuals did not complete all 300 visits on every single night.

All statistical analyses and creation of plots were done in R.

Data availability

All data and analysis code are available online at .....

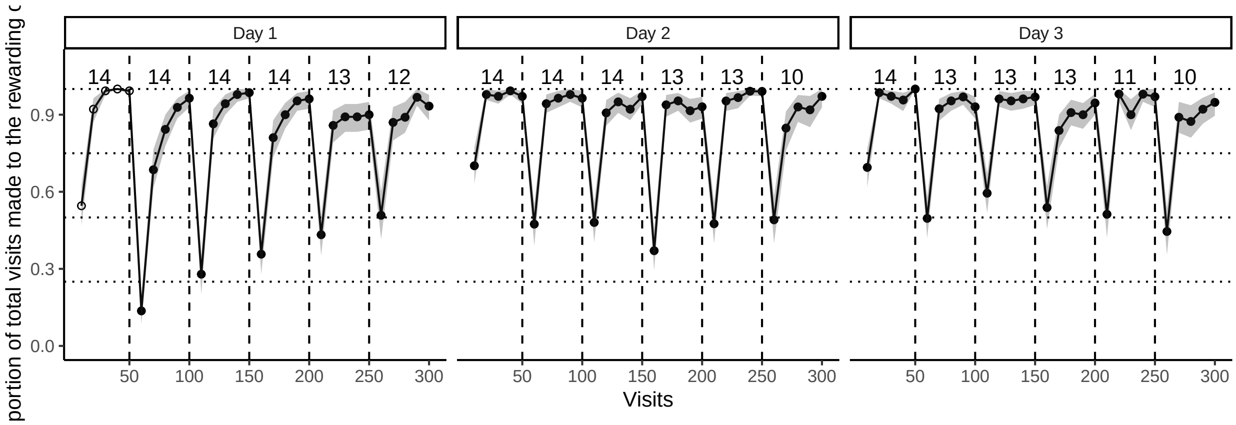
Results

Confirmatory Analyses

a) Bats made more than 90% of their visits to the rewarding option

At the start of the each night, before they had experienced any reversals, the bats made approximately 91% (±SD 7) of their visits to the rewarding flower; immediately following a reversal, this proportion dropped below 50% (**Figure 2**). The proportion of visits to the rewarding flower then increased to 93% (±SD 9) after the first bin of 10 visits following a reversal.

\begin{figure}



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\caption{Figure 2: Average preferences of the bats and the sample size of each block. The first block before the bats experienced any reversals at all is marked with white points, the rest with black points. Shaded areas are 95% confidence intervals}(#fig:Plotting the average behaviour with the sample sizes) \end{figure}

b) Night and reversal block have an effect on the number of perseverative visits following a reversal

Both experimental night and block had a negative effect on the number of perseverative visits made by the bats. As the bats experience more reversals on more nights the number of perseverative visits decreased. The interaction of night and block, however, was positive: the decrease in the number of perseverative visits was smaller as the bats experienced more experimental nights.

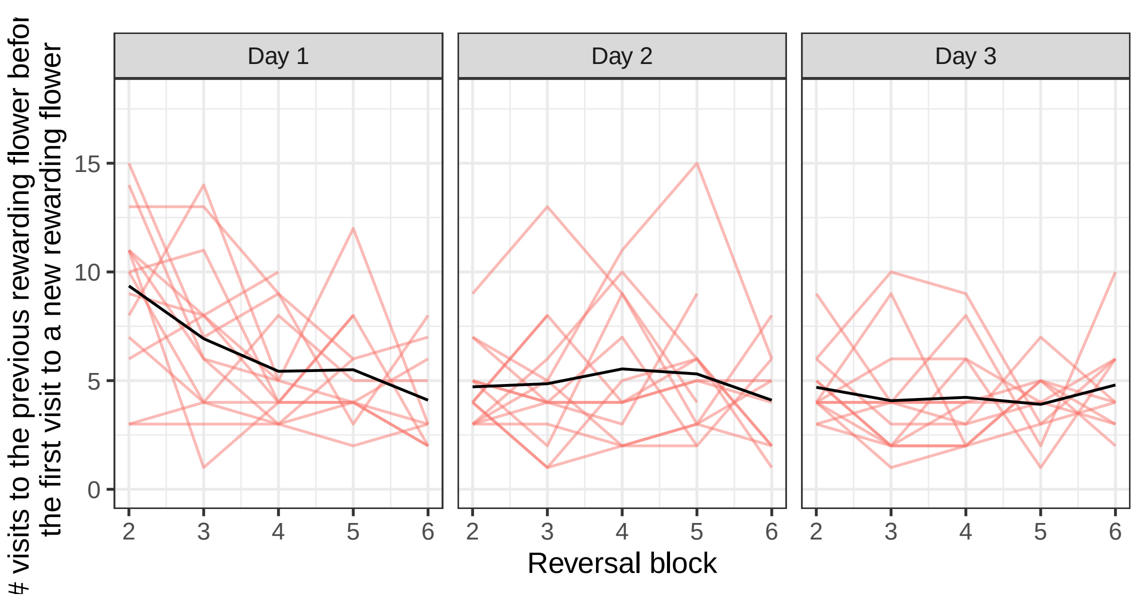

Figure 2: Figure 3: Number of perseverative visits made by the individual bats after a reversal. Red lines are data from the individual bats, black lines are the mean number of visits averaged over bats

Figure 5 shows the fit of the model to the empirical data through sampling from the posterior distribution. The model clearly captures both the negative effect of night and block, as well as their positive interaction.

Table 1: (#tab:Modelling the perseverative visits and plotting the results)Table 1: Results of the random regression model (brms), testing for the effects of day and block (fixed effects) on the number of perseverative visits (dependent variable)

Fixed effect	Estimate	95% Credibility intervals
Intercept	**3.0393791**	**(2.5 , 3.51)**
Day	**-0.5831742**	**(-0.83 , -0.33)**
Block	**-0.2733820**	**(-0.4 , -0.15)**
Day-Block Interaction	**0.0996299**	**(0.04 , 0.16)**

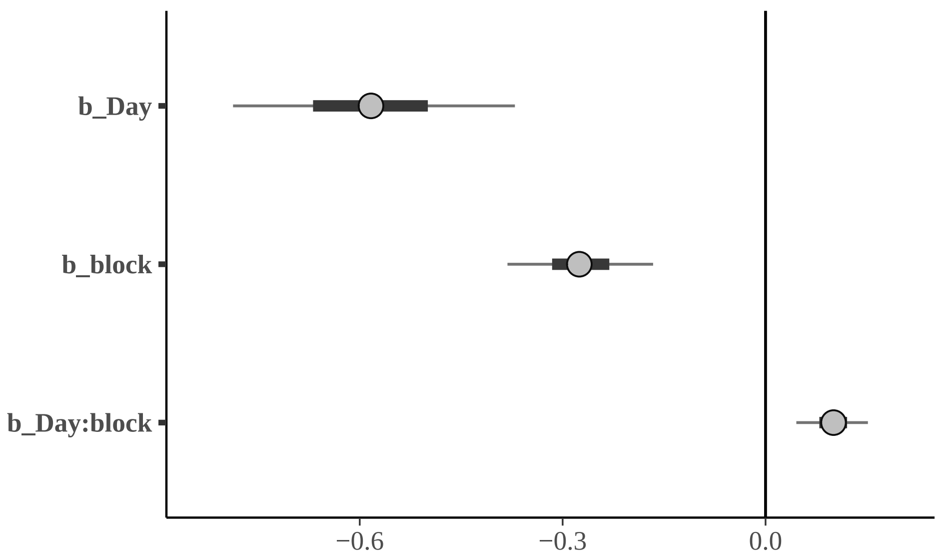

Figure 3: Figure 4: Plot of slope coefficient values of the fixed effects (#fig:Modelling the perseverative visits and plotting the results)

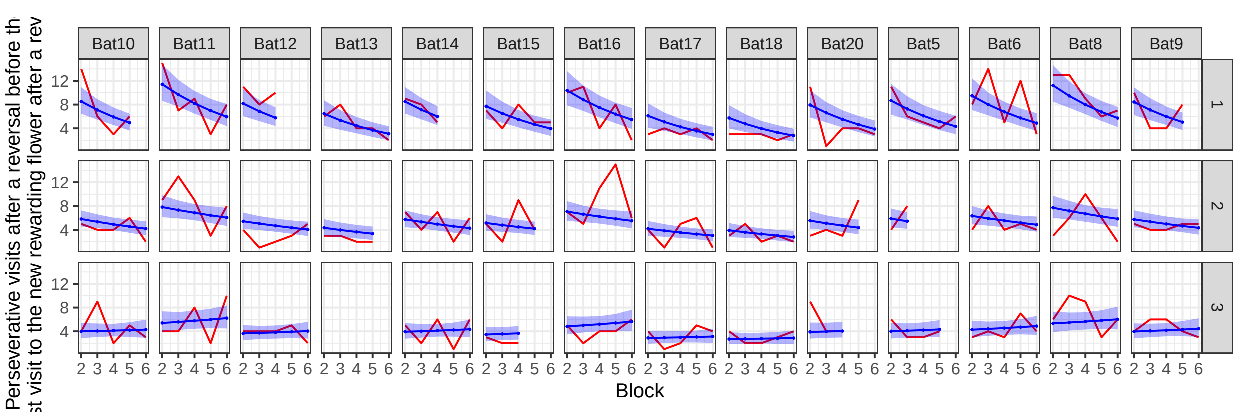

Figure 4: Figure 5: The posterior predictions of the model (blue line), compared with the empirical data from the bats (red line)

Table 2: (#tab:Modelling the proportion of visits to the rewarding flower and comparing two models)Table 2: Comparison of the two models

Model	Difference.in.LOO.estimates	Standard.error
Day and block as both numeric and factor	0.00	0.00
Day and block as only numeric	-108.21	14.14

Table 3: (#tab:Plotting the results of the model of the proportion of visits to the rewarding flower)Table 3: Results of the random regression model (brms), testing for the effects of night, block, bin, night type and block type on the number of perseverative visits (dependent variable)

Fixed effect	Estimate	95% Credibility intervals
Intercept	**-3.2960866**	**(-4.3 , -2.27)**
Day	**0.9383263**	**(0.57 , 1.33)**
Block	**0.4822631**	**(0.33 , 0.63)**
Day Type 1	-0.3893580	(-0.95 , 0.18)
Block Type 1	**1.1983421**	**(0.3 , 2.18)**
Bin	**1.7815207**	**(1.35 , 2.22)**
Day-Block interaction	**-0.1332832**	**(-0.19 , -0.08)**
Block-Bin interaction	**-0.0893776**	**(-0.13 , -0.04)**
Day-Bin interaction	**-0.1843788**	**(-0.34 , -0.03)**
Day Type 1-Block Type 1 interaction	**0.6184034**	**(0.17 , 1.05)**
Block Type 1-Bin interaction	0.0582480	(-0.21 , 0.34)
Day Type 1-Bin interaction	-0.0942867	(-0.35 , 0.16)

Exploratory Analyses

Accounting for the different effect of the first night and the first block of each night in modelling the proportion of visits to the rewarding option resulted in a model with higher predictive accuracy than one that did not account for the first night and first block. (**Table 2**). The results of the model that accounts for the first night and block are presented in **Figure 6**. The night, block and bin variables all had positive slope coefficients, but the interactions of night and block, block and bin, and night and bin all had negative slopes. Though the interactions effects were smaller than the main effects, the 95% credibility intervals of the former did not overlap with 0. Thus, the animals' choice for the rewarding flower increases overall as they experience more and more nights and reversal blocks, and within each reversal block as it progresses, but this 'improvement' in their choice for the rewarding flower decreases within each reversal block as it progresses, within each night as more reversals occur, and within each reversal block as the experimental nights progress. Furthermore, the variable 'block type,' (i.e., whether a block was the first reversal block of the night or not), had a positive slope. The bats made more visits to the rewarding flower before they had experienced any reversal at all. While the 'night type' did not seem to affect the proportion of rewarding flower visits, block type and night type had an interaction effect with a positive slope coefficient: the first block of the first night, before any reversals had ever been experienced even once, had the highest proportion of visits to the rewarding flower compared to any other block on any other night.

Figure 7 shows the fit of the posterior predicted values of this model to the empirical data from the bats. While the model appears to be a good fit overall, it most notably seems to fail to capture the drop in the proportion of rewarding flower visits in the very first bin following a reversal. It is this however that was analysed explicitly in the model fit to the number of perseverative visits; hence this model of the proportion of visits to the rewarding flower was not further expanded to try and capture the behaviour during the first bin of a block.

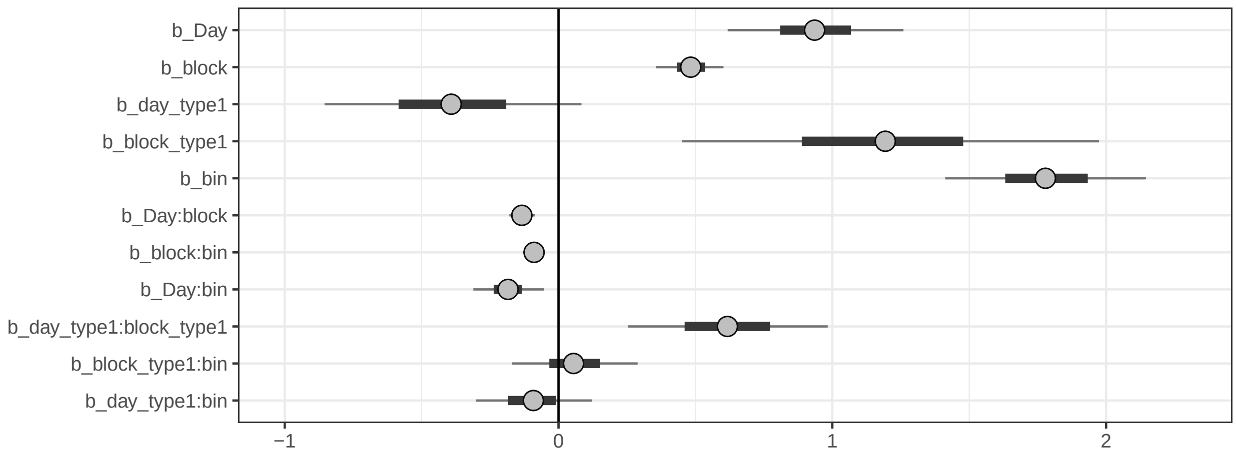

Figure 5: Figure 6: The effect of day, block, bin, day type and block type on the proportion of visits to the rewarding option. Plot of intercept and slope coefficient values of the fixed effects (#fig:Plotting the results of the model of the proportion of visits to the rewarding flower)

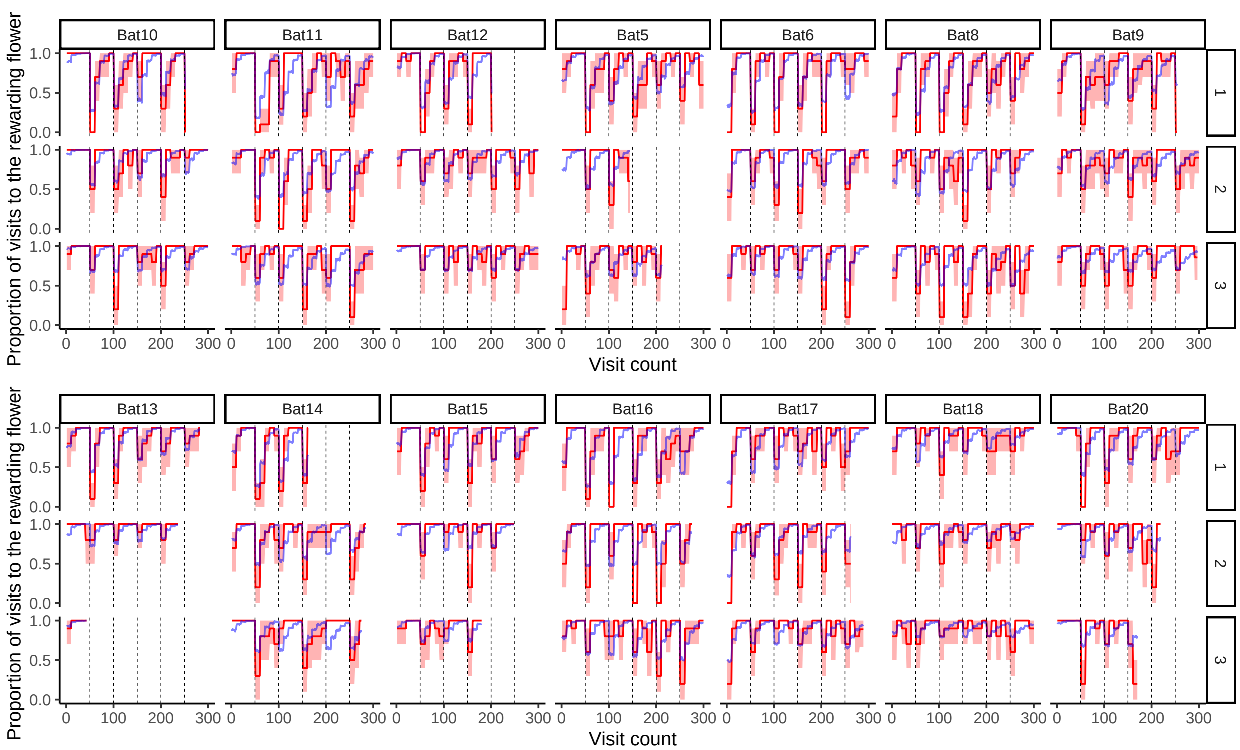

Figure 6: Figure 7: The posterior predictions of the model (blue line), compared with the empirical data from the bats (red line). Reversals are marked as the dotted line. (#fig:Calculating and plotting the posterior predictions)

Supplementary Information

Individual bats' choice behaviour

Each individual visit made by each bat is plotted in **Figure S1**, including the wrongly unrewarded visits so their distribution can be seen clearly.

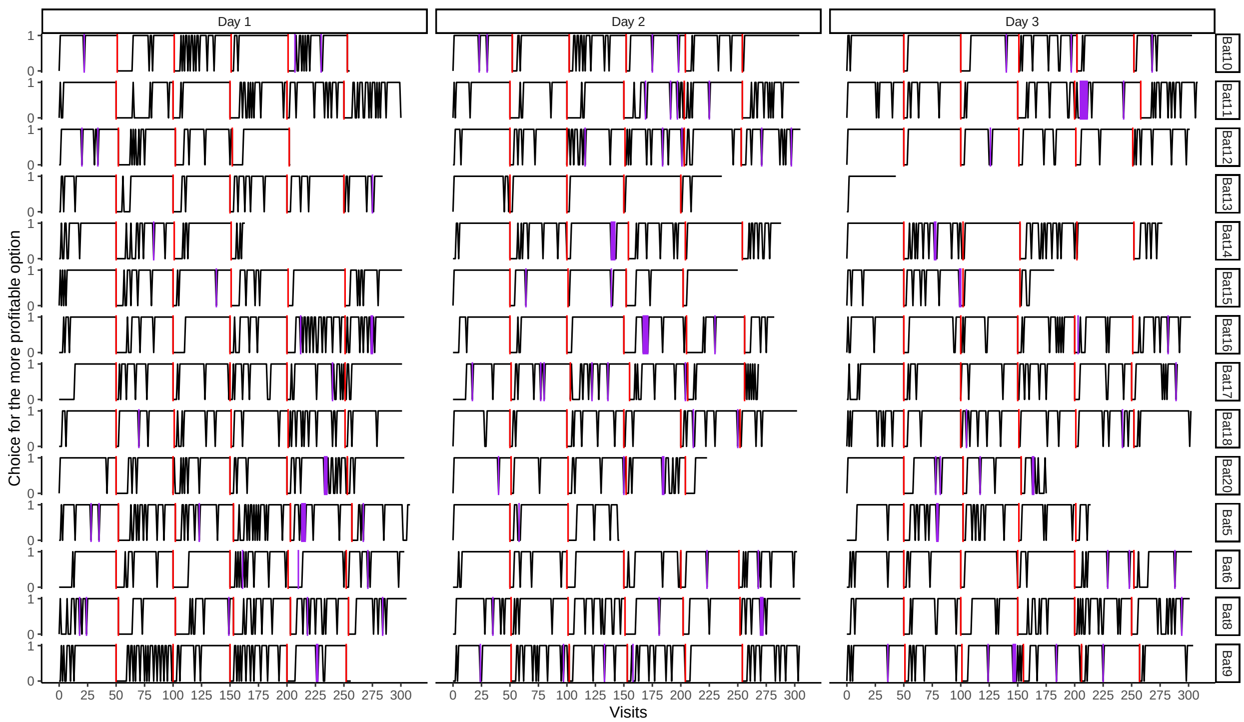

Figure 7: Figure S1: All individual choices of the bats for the rewarding and non-rewarding options. The black line is the choice for either the rewarding flower or unrewarding flower, marked as 1 or 0 on the Y-axis respectively. The red lines indicate when a reversal took place and the purple areas mark the wrongly unrewarded visits
(#fig:Plotting all the individual choices)

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