Tortoises develop and overcome position biases in a reversal learning task

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Abstract

The capability of animals to alter their behaviour in response to novel or familiar stimuli, or behavioural flexibility, is strongly associated with their ability to learn in novel environments. Reptiles are capable of learning complex tasks and offer a unique opportunity to study the relationship between visual proficiency and behavioural flexibility. The focus of this study was to investigate the behavioural flexibility of red-footed tortoises and their ability to perform reversal learning. Reversal learning involves first learning a particular discrimination task, after which the previously rewarded cue is reversed and then subjects perform the task with new reward contingencies. Red-footed tortoises were required to learn to recognise and approach visual cues within a Y-maze. Once subjects learned the visual discrimination, tortoises were required to successfully learn 4 reversals. Tortoises required significantly more trials to reach criterion (80% correct) in the first reversal, indicating the difficulty of unlearning the positive stimulus presented during training. Nevertheless, subsequent reversals required a similar number of sessions to the training stage, demonstrating that reversal learning improved up to a point. All subjects tested developed a position bias within the Y-maze that was absent prior to training, but most were able to exhibit reversal learning. Red-footed tortoises primarily adopted a win-stay choice strategy while learning the discrimination without much evidence for a lose-shift choice strategy, which may explain limits to their behavioural flexibility. However, improving performance across reversals while simultaneously overcoming a position bias provides insights into the cognitive abilities of tortoises.

1. Introduction

Animals living in complex environments exhibit an enhanced capacity to learn, can rapidly alter their behaviour under different contexts, have more versatile responses to fluctuating resources (Gaalema 2011; Bond et al. 2007), and are flexible in adopting spatial and non-spatial abilities as the environmental surroundings demand (Day et al. 1999). This ability to alter behaviour by developing new responses to novel stimuli or by modifying responses to familiar stimuli is known as behavioural flexibility (Leal and Powell 2011). Animals that are opportunistic feeders, such as many reptiles, must demonstrate behavioural flexibility in their foraging ability (Moskovits and Bjornadal 1990), and may learn to discriminate between visual cues and establish orientation preferences while foraging. Indeed, the highly developed visual system of tortoises allows them to distinguish different food items, such as fallen fruit and flowers that vary depending on the season (Moskovits and Bjornadal 1990). Differentiating between various visual cues requires that animals have the appropriate sensory capacity (Delius and Delius 2006); tortoises have excellent colour vision and are capable of visual discrimination of images of familiar objects (Wilkinson and Huber 2012; Wilkinson et al. 2013).

Investigations into reptile cognition and learning have used reversal learning tasks to assess the flexibility of their behaviour (Day et al. 1999; Day et al. 2003; Gaalema 2011; Holmes and Bitterman 1966). Reversal learning is a form of learning where a subject that has learned a discrimination task must subsequently learn to respond to the previously non-rewarded stimulus-reward pairing (Holmes and Bitterman 1966). Reversal learning therefore involves switching choice strategies, at least during the initial unlearning period. A win-stay, lose-shift strategy might be used to be successful in reversal learning, where subjects only repeat the previous choice when positively reinforced (Mackintosh et al. 1968; MacPhail 1982; Davey 1989).

Research is limited on visual discrimination tasks in reptiles, particularly involving the reversal learning of visual tasks (Wilkinson and Huber 2012; Wilkinson et al. 2013). Monitor lizards have shown improved performance across successive reversals (Galeema 2011), while painted turtles improved performance within an open apparatus visual and spatial discrimination task across 10 reversals (Holmes and Bitterman 1966), and red-footed tortoises have shown strong capacity for visual discrimination but limited capacity to improve learning with serial reversals (Smith 2012). In the study by Smith (2012), tortoises were tested in outdoor enclosures where cues from outside the arena were not be controlled, the reinforced stimulus was not alternated in terms of position, and tortoises were tested with experimenter present. Thus, it is not certain to what extent inadvertent cuing (*i.e.,* uneven lighting from sun and shade, extra-maze cues, Clever Hans effects), helped or hindered choice or performance.

Learning novel visual tasks depends on an animal's behavioural flexibility in processing visual stimuli, but when visual discrimination tasks also involve or require the use of spatial and locomotor abilities, as is inescapable in some experimental scenarios, they could be influenced by lateralisation, whether innate or through experience. Cerebral lateralisation, the difference in the structure or function between the left and right sides of the brain (Rogers 2000), can influence the lateralisation of visual and motor functions. Lateralisation in an animal's vision (*i.e.,* visual asymmetry) may influence motor behaviours to become preferentially executed in a particular direction (Gunturkun et al. 2000). The latter has been observed in fish predatory behaviour (De Santi, 2001) and in their turning behaviour in a T-maze (Facchin et al. 1999). Behavioural lateralisation has been found in reptilian detour behaviour (Csermely et al. 2010), escape behaviour (Bonati et al. 2010), predatory behaviour (Bonati et al. 2008), righting behaviour (Stancher et al. 2006), responses to mirrors (Sovrano et al. 2017), and in brightness discrimination tasks requiring movement (Spigel 1963).

Behavioural lateralisation may be advantageous in complex tasks involving visual and spatial components. For example, lateralized birds and fish have higher efficiency than non-lateralized individuals in learning tasks requiring coordination of visual and spatial components (Magat et al. 2009; Sovrano et al. 2005). Further, in coordination tasks, the strength of behavioural lateralisation has also been shown to have a positive relationship with performance in parrots (Magat et al. 2009), chimpanzees (McGrew and Marchant, 1999), and marmosets (Piddington and Rogers 2013). Lateralisation could result in greater cognitive ability through enhanced simultaneous processing (Rogers 2000; Vallortigara and Rogers 2005), where each hemisphere can take charge of different subtasks, resulting in parallel processing (Rogers et al. 2004). This allows lateralized individuals to have an increased capacity to handle two simultaneous tasks and may particularly help prevent simultaneous initiation of incompatible responses in animals with laterally placed eyes (Vallortigara and Rogers 2005).

Learning tasks can also strengthen pre-existing side or hand biases. In numerous primates, complex tasks show more hand bias compared to simpler tasks that do not require coordination (Hopkins and Rabinowitz 1997; Meguerditchian et al. 2010; Vauclair et al. 2005; Hopkins, 1995). Moreover, stronger individual hand preferences in gorillas have been observed in coordination tasks involving simultaneous visual and spatial components compared to simpler tasks (Fagot and Vauclair 1988a and 1988b; Spinozzi et al. 1998). Additionally, early studies on visual discrimination in turtles provide insight into how incurred position biases impact learning; subjects showed individual turning preferences while learning different visual tasks (Casteel 1911; Spigel 1963), while lower performance occurred in subjects with strong turning preferences (Spigel 1963). When tortoises are subjected to navigation of a simple maze, position preferences may arise during exploratory behaviour, or because movement in tortoises requires coordination simply to maintain balance (Jayes and McNeil-Alexander 1980; Gans et al. 2011).

In a study on spatial task performance, an individual red-footed tortoise revealed a tendency to turn consistently in the same direction when extramaze cues were not present, allowing it to avoid previously occupied arms in a radial-arm maze (Wilkinson et al. 2009) seemingly employing a strategy allowing it to visit each arm only once. Position biases within open arenas have also been shown in lizards (Day et al. 1999).

The primary objective of this study was to understand how behavioural flexibility enables red-footed tortoises (*Chelonoidis carbonaria*) to change choice strategies during a reversal learning task requiring visual and motor coordination. Since previous research in red-footed tortoises demonstrated a limited capacity for behavioural flexibility (Smith 2012) but used a reversal learning procedure that may have supplied inadvertent cuing, we incorporated a visual discrimination and motor coordination procedure within a Y-Maze. We hypothesized that red-footed tortoises would associate visual cues with successful acquisition of a food reward and predicted that if reversal learning was occurring independent of position bias, fewer sessions would be required to reach the learning criterion for subsequent reversals.

2. Methods

2.1. Subjects

For the present study, a total of 5 adult female captive-bred (~6 years of age) red-footed tortoises were used. Animals were identified using a two-colour code system (non-toxic paint delineated by two lines on the marginal scutes of the carapace) for each tortoise: blue/blue (BB), purple/purple (PP), red/red (RR), red/blue (RB), and purple/blue (PB). Subjects were experienced in moving within the Y-maze guided by food rewards but had no prior experience with reward association; a pilot study examining population level position bias involved testing tortoises within the Y-maze to approach identical visible food rewards placed at the end of both arms. Twelve animals (N=7 were temporarily available from a private collection in addition to the 5 used throughout the remainder of the study) were given 18 trials and their arm choice (left or right) scored and compared to that expected at random. All general test procedures in the pilot study follow the descriptions below. All procedures, husbandry, and experiments complied with the Canadian Council of Animal Care guidelines and were approved by the local animal care committee (AUP# 12-01-03).

2.2. Learning and Test Apparatus

Stimuli consisted of differently coloured shapes, generated by coloured construction paper against white paper background, held within plastic cardholders that were placed at the ends of a Y-maze (Figure 1). Subjects were placed in a free arm of a Y-maze (arm dimension 34 cm wide x 70 cm long; wide enough to allow tortoises to turn around) behind a sheet of plexiglass facing towards the other two arms to allow prior investigation of the different visual stimuli. The outside of the Y-maze was completely surrounded by black cloth in order to remove the potential use of extramaze cues by the tortoises. The investigator withdrew during the trials to eliminate experimenter cues and watched the tortoise from a live video-feed. Although evidence from the pilot study suggested no population or individual level lateralisation in simple movement tasks within the Y-maze, positive stimulus and starting arm placement were randomly arranged throughout all trials to avoid unintended visual or spatial cues from influencing learning.

2.3. Experimental Procedure

The discrimination and learning task involved rewarding subjects with food for approaching the positive stimulus, while no punishment, except a lack of reward, was administered for approaching the neutral (*i.e.,* non-rewarded) stimulus. Food rewards included a single piece of honeydew melon or strawberry. The task (*i.e.,* trial) consisted of a 30 second period behind the transparent barrier to allow tortoises time to investigate the different stimuli (approximately 1 meter from the tortoise but within its visual field). Once the barrier was removed, the tortoise navigated to one arm, often pausing at the junction point, approximately 70 cm from the stimulus. A food reward was given on the end of a wooden dowel once the subject was ~6 cm from or attempted to bite the positive stimulus. Trials were terminated if tortoises showed no signs of movement within three minutes (the 99^{th} percentile to decision was 95 s); these trials were not scored and were only observed during the initial familiarisation session. The starting arm in each trial was randomized, and the arm that consisted of the positively reinforced stimulus was randomly selected to be either the left or the right arm relative to the starting arm; the positive stimulus was placed no more than three consecutive times in the same direction (*i.e.,* LLL or RRR) to reduce the possibility of a positional preference also being reinforced. A GLM was performed to verify that placement was random with respect to Stage ($p=0.62$) and Session ($p=0.95$) and Stage*Session ($p=0.79$). Following the completion of a trial, the tortoise was removed from the Y-maze and placed in a high-walled container for ~60 seconds before starting the next trial. Tortoises choosing the positive stimulus were allowed time to eat the reward, while tortoises choosing the neutral stimulus were immediately removed to the holding container. Subjects performed between one and four sessions per day, with each session totalling 10 trials. Between 2 and 5 subjects were used for experimentation on a given trial day, and experiments were conducted over a 6-month period.

The entire experimental procedure consisted of three phases: familiarisation, pre-training, and the experimental phase. The familiarisation phase was used to assess that all tortoises would navigate from the starting arm to one of the choice arms and approach the stimulus. Since all subjects were already experienced in performing visual tasks in the Y-maze the familiarisation phase also served to ensure that experimental tortoises would perform prior to the pre-training stage; we had previously observed that naïve tortoises would not explore the Y-maze within a 3-minute period unless a food stimulus was present. The first stage of the familiarisation phase involved only one stimulus (different from any used in the subsequent experiments) with food placed 3 cm in front of the visual stimulus placed at the end of an arm, chosen at random. Completion required five consecutive correct choices, a task that was completed readily by all tortoises within the first 5 trials due to the presence of food. The subsequent stage of familiarisation was the same as the first except the food reward was only given to the subject once the correct choice was made. Completion of this stage also required five consecutive correct choices. Since the familiarisation phase revealed individual food preferences among the tortoises, we used this phase to customise rewards for each tortoise. Once tortoises succeeded with familiarisation, they proceeded to the pre-training phase, which involved introducing a non-reinforced stimulus to the second arm. Subjects were then required to reach a criterion of 16/20 (*i.e.,* 80% correct) during a full test of two sessions in order to move onto the experimental phase. The experimental phase consisted of five stages: a training stage (*i.e.,* acquisition stage described in Smith 2012), followed by 4 reversals (R1, R2, R3, R4). Acquisition of the task during the training stage involved introducing a new set of stimuli from those used during the pre-training and familiarisation phases. After successful training, subjects had to reach criterion (16/20 over two successive sessions) in each of 4 serial reversals. Serial reversals consisted of presenting the same two stimuli to each subject in each reversal, with the positive and neutral stimuli switching reward contingencies once the subjects reached the advancement criterion.

2.4. Data Analysis

Data from the familiarity and pre-training sessions were not incorporated into statistical analyses, although pre-training data are reported for comparison. Statistical analysis on the learning experiments was performed using Linear Mixed Models (response variables: trial time and trials to reach learning criterion) and Generalised Linear Mixed Effects Models (binomial response variable: correct *vs*. incorrect trial outcome with logit link) using R (R Core Team, 2015), with the *nlme* (Pinheiro et al. 2015) and *lme4* packages (Bates et al. 2012). Parameters tested were Stage (factor with 5 levels: Training, R1-R4), Session (as a numerical covariate), and Direction (factor with 2 levels: Left, Right), where appropriate, along with all two-way interactions with Stage. These parameters were chosen to examine changes across the learning paradigm (i.e. reversals) and within a given learning stage, and to account for the influence of directional biases on learning. In all cases, subject (Tortoise ID) was modelled as a random intercept and slope with respect to Session. Model residuals were assessed for normality (where appropriate) and equal variance with respect to predictors. We present model coefficients with *P* values as measures of support, calculated using parametric bootstrapping based on 10000 simulated re-samplings of the observed dataset to allow the empirical distributions of effects to be estimated (Zuur et al. 2009). Effect plots were used to visualise the model fits (±95% confidence intervals) with respect to the parameter of interest, holding other parameters to their average value, using the *effects* package in R (Fox, 2003). For GLMMs, the effects for the fixed variables are transformed to the probability of making a correct choice due to the binary response of the task. Position bias was further examined in two ways, using the terminology from Martin and Bateson (1986), as relative lateralisation:

$$L_r = \frac{(N_r - N_l)}{(N_r + N_l)}$$

where N_r refers to the number of right turns, and N_l refer to the number of left turns (L_r: -1 = left and +1 = right) over a session or learning stage. Absolute lateralisation (L_a: where 0 = none and +1 = full bias), or the strength of position bias within an individual, was calculated as:

$$L_a = \frac{|N_r - N_l|}{(N_r + N_l)}$$

Given their apparent non-normal distributions, absolute lateralisation data were analysed using a Wilcoxon Signed Rank test compared to the null expectations of a binomial response variable with p=0.5, verified through bootstrapping 10000 samples.

During the pre-training, training, and reversal learning trials, we also examined the learning strategy across sequential trials to ascertain the extent to which tortoises adopted a "win-stay" and/or "lose-shift" strategy, employing a Markov chain approach as described in Martin and Bateson (1986). For each pair of subsequent trials, one of 4 patterns (*i.e.,* WW, LW, WL, LL) is possible: win-stay, lose-shift, win-shift, lose-stay. For example, if the tortoise chooses the positive stimulus (win) on trial 1, selecting the same stimulus on trial 2 would be classified as "stay", and thus that pair of trials categorised as a "win-stay" pair. Given only 4 possibilities (2^2=4), a randomly choosing or a complete position-biased animal would exhibit each category 25% of the time, since the positive stimulus was randomised with respect to direction. This approach allowed us to examine which particular visual cue strategy the tortoises employed during the learning process within each stage. To learn rapidly and demonstrate flexibility, they should employ both a "win-stay" and "lose-shift" strategy, although given that the neutral stimulus provided little reinforcement, we might not expect the "lose-shift" strategy to be used. The strategies were summarised for the first and last 20 trials (*i.e.,* 2 sessions, leading to 19 pairs each) during a learning stage in order to focus on learning, rather than carry-over memory effects between sessions.

3. Results

3.1. Learning task and criteria

All tortoises successfully learned the visual discrimination during the pre-training and training stage. Tortoises reached the learning criterion in the pre-training stage within 58 (sd=15) trials. After the subjects reached criterion in the training phase, they then performed reversal learning in 4 subsequent reversals. Four out of the 5 subjects finished all 5 stages; subject RR only reached criterion for the initial training and reversal 1. In reversal 2, RR was unable to reach criterion after 300 trials. Two sets of visual stimuli were used throughout the experiment (Figure 1), with no discernible differences, as tortoises from both sets proceeded through the reversal learning process. Each trial required that tortoises navigate the maze and make their decision to approach a stimulus. The time to complete the task was recorded in every trial (on average 22 seconds), and the influence of Stage and Session during the experimental phase and their two-way interaction examined using LMM (Supplementary Table 1; Supplementary Figure 1). Stage had no significant effect, while trial completion time showed a general trend to decrease across session (~0.6 seconds/session; P=0.0058). An interaction between Session and Stage (Supplementary Table 1) appeared to be driven by the fact that trial time ceased to decrease across sessions by reversal 1, 2, and 3 (*P* values ~0.04 for the interaction terms). The number of trials required to reach criterion was significantly affected by learning stage (*P*=0.032), primarily driven by the difficulty of the first reversal (Figure 2); subsequent reversals required similar number of trials to the training stage.

3.2. Nature of reversal learning

The probability of a correct choice rose across sessions, approaching the learning criterion of 16/20 (*i.e.,* 0.8) in a stage-dependent manner (Figure 2). The interaction between

Stage and Session was driven by the lower rate of rise during the first two reversals, especially during R1 (Figure 2), due to the low starting probability in the earliest sessions. The direction (left vs. right) the tortoise moved to perform the task correctly influenced the probability of choosing the positive stimulus (OR=3.2 for moving right: P value < 0.0001), revealed also through interactions between Direction and Stage (P values ranged from 0.0002 to 0.07; Supplementary Table 2). Tortoise PB showed the most dramatic improvement across reversals, taking 230 trials to reach criterion in R1 and 60 trials to learn the visual discrimination in R3 (Figure 3). RB was the only subject to not experience difficulty in R1, demonstrating high proficiency across all stages, although was flexible in switching from slight right position bias toward a left position bias by the 4th reversal. Qualitatively, tortoises had a high level of attention during movement within the Y-maze, highlighted by side-to-side head movement when approaching the Y-junction (see Supplementary Videos 1-4). This was occasionally accompanied by a decrease in movement speed and pausing at the Y-junction. In addition, tortoises occasionally exhibited correct choices after initially moving along the incorrect arm but immediately turning around (*i.e.,* position errors; Day et al. 1999). Individual variation in task completion time was evident (Figure 3), although there was little apparent change across stages.

The win-stay approach appeared to be the primary choice strategy employed for the learning task, with little to no contribution of a lose-shift strategy, which remained close to random chance within the beginning and final sessions of a learning stage (Figure 4). At the beginning of each reversal the lose-stay choice strategy was elevated above the levels observed during the pre-training or training stages (Figure 4), although the lose-stay choice strategy was reduced almost to 0 within the last session of each learning stage.

3.3. Learning in the context of position bias

Prior to the learning trials, a pilot study revealed no population level position bias (GLMM Odds Ratio Right versus Left = 1.07 (95%-CI: 0.79-1.47; $P = 0.63$), and no evidence of individual level lateralisation (*i.e.,* position bias), as quantified through a Wilcoxon Signed Rank test ($L_a = 0.24$ *vs.* μ=0.185, N=12, V=66, $P = 0.11$). The mean null expectation of L_a (μ) for 18 random draws from a binomial distribution with p=0.5 is 0.185 (verified through bootstrapping), not zero. In contrast to the pilot and the pre-training experiments, the tortoises in the learning trials exhibited individual variation in position bias (Figure 3), which was enhanced in certain individuals across learning stages ($L_a = 0.643$, 95%-CI: 0.547-0.752, $P<0.0001$). This change in L_a reflected individual performances when turning to the left or right changed across stages (Supplementary Figure 2). There was an overall trend toward higher L_a with respect to Stage, which may partially be explained by the stronger bias observed in the first 20 trials of R3 and R4 (Figure 5). This position bias, however, decreased within a learning stage, a necessity to reach the learning criterion. A strong individual position bias was also associated with increased difficulty in the task (Supplementary Figure 3). Tortoise RR only completed training and reversal 1, with RR repeatedly turning left until nearing criterion in reversal 2. Tortoise RB also developed extreme position bias in R4, showing 78 successive trials of turning left, but reached criterion within the last 2 sessions of 10 sessions almost immediately after it overcame this position bias. During the training stage and in some of the later reversals, two tortoises were more successful at choosing the positive stimulus if they were moving toward the right (Supplementary Table 2; Supplementary Figure 2).

4. Discussion

Red-footed tortoises were capable of successful reversal learning of a reward association in a navigational task. Given the difficulty of unlearning the reward association, it is not surprising that past studies have shown a higher number of trials required to reach learning criterion in the first reversal (Holmes and Bitterman 1966; Day et al. 1999). Red-footed tortoises have previously shown little improvement with serial reversal learning (Smith 2012), but under conditions where inadvertent cues were not controlled and where a navigational component was not required. The difficulty of reversal learning is substantiated in the present study where the average number of trials to reach criterion in the first reversal was significantly higher than in the training stage. Although there was no obvious improvement in the trials to reach criterion in successive reversals compared to the training stage, there was a steeper rise in correct responses (*i.e.,* learning) within R3 and R4, suggesting potential for behavioural flexibility. Previous research on reversal learning in turtles has shown improvement in serial reversal learning of a visual discrimination (Holmes and Bitterman 1966). In contrast, tortoises in the present study needed to successfully coordinate movement in a maze while differentiating between visual cues at a distance. Furthermore, improvement in serial reversal learning requires a capacity to generalise learning strategies. MacPhail (1982) predicted that improvement across reversals is not expected until the second reversal, at which point a "win-stay, lose-shift" strategy would be more likely employed. In the present study, a win-stay approach was important for reversal learning, although red-footed tortoises showed little evidence of employing a lose-shift strategy, as they reached the learning criterion, although this is presumably related to the low error rate in the last two sessions. At the beginning of each reversal, the lose-stay strategy was high, as the previously positive stimulus continued to interfere with learning; it was, in part, due to a suppression of the lose-stay tendency that tortoises achieved the learning criterion during subsequent reversals (Figure 4).

Nevertheless, tortoises not only demonstrated familiarity in the learning task by showing improvement during a reversal learning stage, based on the trial time improvements and high trial completion rates, subjects were proficient and eager to navigate the Y-maze early in experimentation, as seen by the decrease in trial time in the training stage. The time taken per trial reached a plateau as turtles learned how to navigate a maze (Tinklepaugh 1932; Spigel 1966) or even a dual-choice chamber (Spigel 1963). The fact that trial completion time did not change across learning stages may reflect the strong individual differences in movement speed and decision-making.

Although not present at the outset, a position bias developed, increasing in strength in later reversals. One explanation for the position bias relates to the difficulty of the reversal learning paradigm itself, which may have reinforced minor position preferences. A position bias developing in a task that randomises the position of the positive stimulus between arms of the maze may occur because Y-maze navigation occurs simultaneously with the visual task, as opposed to previous reversal learning research which involves only visual discrimination without a navigational component (Smith, 2012). Since the same tortoises had no position bias during simple navigation of the Y-maze in a pilot study, the developed position bias compares with prior research showing stronger individual biases in complex tasks compared to simple tasks (Fagot and Vauclair 1988b). The only comparable example of position bias in Testudines would be a turning preference in a dual-choice chamber (Spigel 1963), which may have been a result of increased motor coordination demands while learning visual discriminations. Increased familiarity of the task may also have contributed to the strengthened position bias. Experience in a simple reaching task leads to a strengthened hand preference in primates (Lehman 1980).

Other research on rats and fish has shown that the strength of bias is flexible with repetition over several experimental days, with a change in turning bias strength during simple navigation of a T-maze and during escape behaviour (Rodriguez 1992; Cantalupo 1995).

The observed position bias in red-footed tortoises may also have been generated by preferential eye use in the form of visual asymmetry, similar to preferred eye use in fish, which directly influences the swimming direction in a T-maze (Facchin et al. 1999). In common wall lizards, left eye preference occurs during maze navigation (Bonati et al. 2010, 2011; Csermely et al. 2010). The same species also show faster turning in either direction while limited to the use of the left eye (Bonati et al. 2013). During navigation of the Y-maze, we sometimes observed side-to-side head movements when tortoises approached the junction. The lateral position of the eyes in tortoises is commonly associated with monocular viewing when focusing on an object (Vallortigara et al. 1999). Alternating head orientation is needed for scanning the environment separately (Deckel 1995), and task allocation for each eye may be crucial in overcoming limited interhemispheric communication required for complex maze tasks especially where stimuli are far apart, since the absence of a corpus callosum in reptiles limits communication between hemispheres (Deckel 1995). Thus, head-turning also allows for time to process visual discriminations, while allowing increased viewing with the preferred eye (Vallortigara et al. 1996). Therefore, since turning bias appears to be driven by eye preference (Facchin et al. 1999; Vallortigara et al. 1996), task allocation with monocular viewing may help explain the strong position bias in red-footed tortoises navigating the Y-maze. Indeed, when the stimulus was on the right side of the tortoise, there was a strong trend toward improved performance in at least 2 of the tortoises, especially in the later reversals (Supplementary Figure 2). The potential disadvantage of increased head-turning is that it is a time-consuming behaviour (Franklin, 2001). Although head-turning might help tortoises learn the visual discrimination through monocular viewing, the Y-maze forced a choice to be made prior to reaching the stimulus and receiving the reward; this likely made it difficult to efficiently process both visual stimuli before passing the Y-junction after which the tortoises were nearly committed to one side, which may help to explain the occasional position errors observed.

The presence of a position bias made success in a two-choice learning task more challenging, as witnessed by the failure of one subject (RR) to complete the experiment, remarkably having learned to become completely left-turn biased; such individual differences are not uncommon when performing difficult tasks (Marchant and Steklis 1986; Bonati et al. 2008). We also observed occurrences of correct choices when tortoises initially advanced into the incorrect arm and then immediately turned around to enter the correct arm, described as position errors by Liu and Day (2015). Although not quantified, these position errors appeared more commonly when tortoises were about to reach criterion, signalling the difficulty of turning towards the unbiased direction, or simple navigational errors.

5. Conclusions

Red-footed tortoises successfully demonstrated reversal learning of a visual discrimination, however they also adopted a position bias that was not present at the start of the study, which impacted their capacity for behavioural flexibility. Despite this bias, reversal learning still occurred; it appears that there was a higher initial rate of "unlearning" (*i.e.,* fewer initial "lose-stay" errors) of the previous positive stimulus in the later reversals which allowed tortoises to overcome the position bias. Coordination of these tasks provides insights into the cognitive abilities of red-footed tortoises, which may ultimately relate to their discriminating abilities to forage and remember locations of fluctuating food resources in their natural environment.

Compliance with Ethical Standards

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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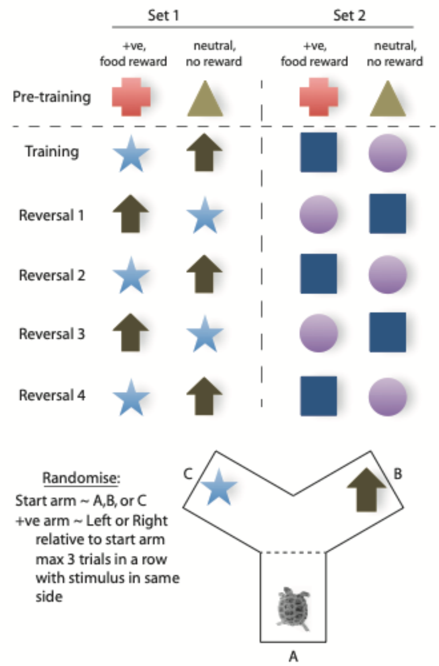

Figure 1. Visual representation of the learning paradigm, showing the respective stimuli presented to the subjects, and the Y-maze apparatus. Stimuli used during the pre-training phase were different from those used in the experimental phase (training and reversal stages). Two sets of stimuli were utilised, with the positive (*i.e.,* reinforced) stimulus alternating within a set between reversal stages. Animal starting placement and stimulus placement relative to starting arm were randomised within the 3 arms of the Y-maze throughout all trials, according to a schedule that avoided >3 trials in a row in the same direction.

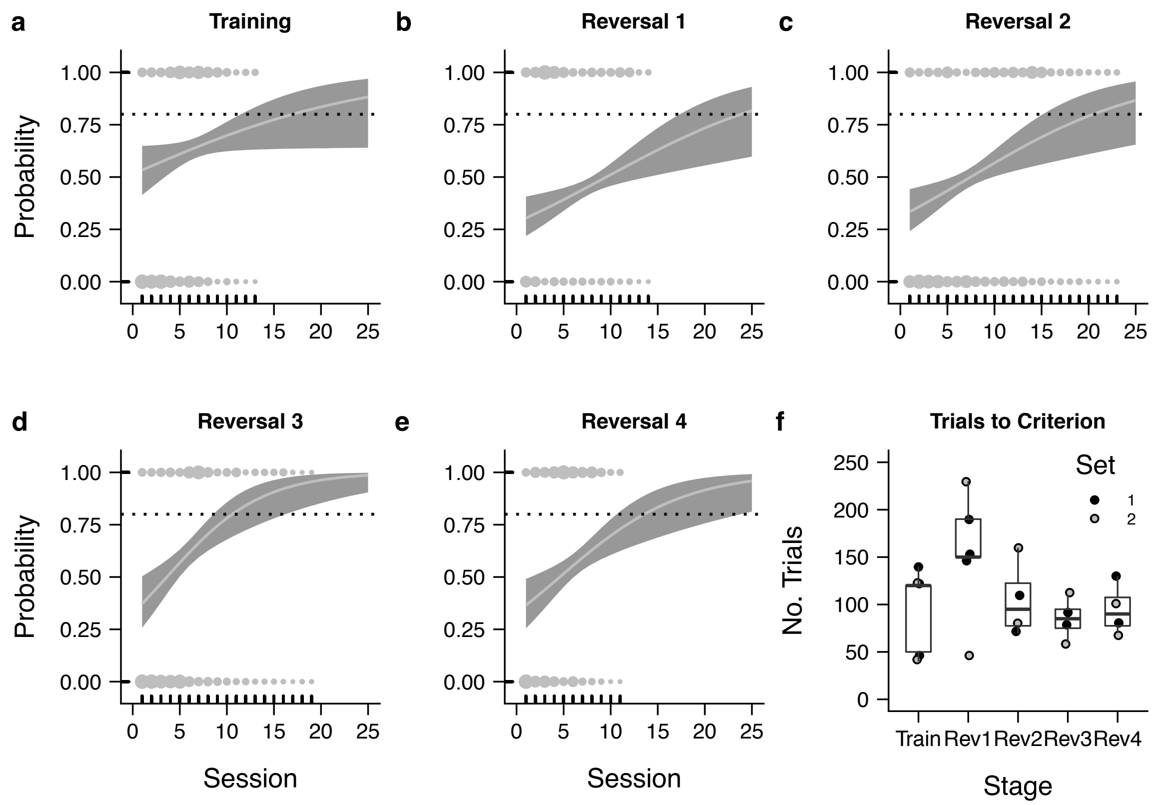

Figure 2. Learning response curves during training and serial reversals of a reinforced visual stimulus in red-footed tortoises, assessed by the probability of correct choices occurring within a session of 10 trials. Grey points depict the response variable, where symbol size depicts the number of observations (*i.e.,* trials). Tortoises progressed through stages after they reached the learning criterion (16 successful choices over 20 trials occurring in adjacent sessions; horizontal dotted lines). The training stage is shown in panel a, while reversals 1 through 4 are depicted in panels b-e; marginal effect display plots (accounting for other influences in the model) are shown with model 95% confidence limits depicted in grey shading. The number of sessions to reach the advancement criterion (panel f) was significantly (P = 0.032) higher during the first reversal but returned to the level observed during the training stage for subsequent reversals. Set 1 and 2 refer to the learning sets depicted in Figure 1.

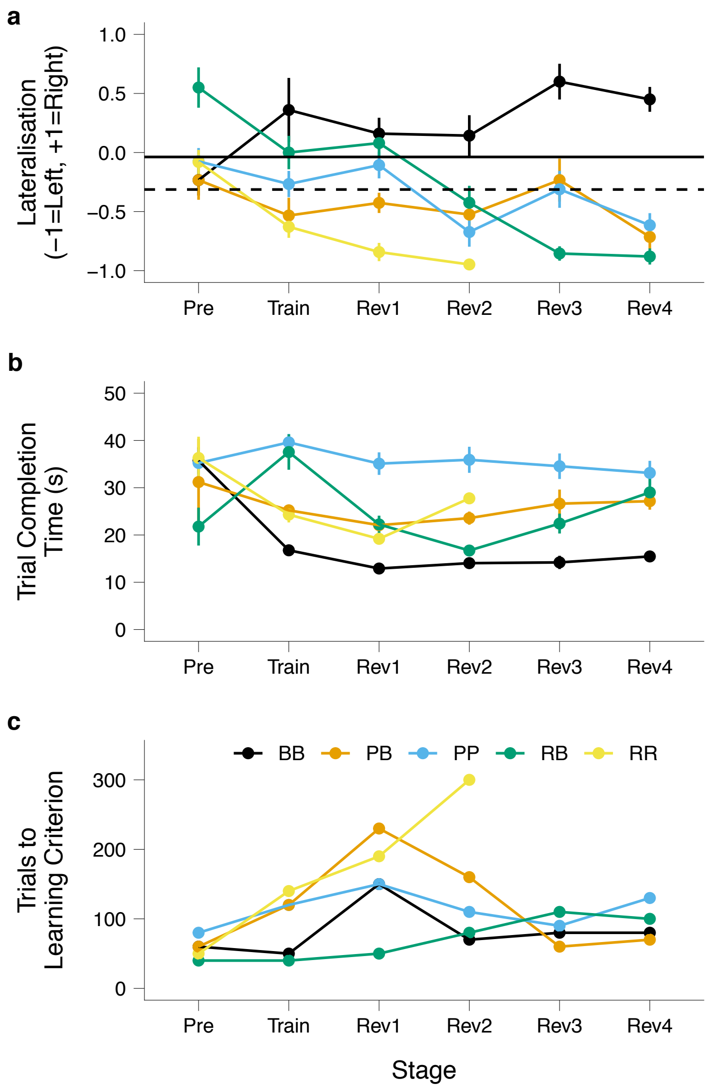

Figure 3. Position bias measured by the Relative Lateralisation indices (individual's mean ± se) in red-footed tortoises during serial reversal learning are shown in panel a (pre-training, training, reversals 1-4). Solid horizontal line represents the degree of lateralisation present in pilot experiments prior to any learning trials (not significantly different from random chance; *P*=0.11). Dotted lines represent the mean response over the entire experimental period (from training and all 4 reversals). Task completion time for each individual across stages is shown in panel b. The total trials required to reach the 80% learning criterion is depicted in panel c for each tortoise for each learning stage.

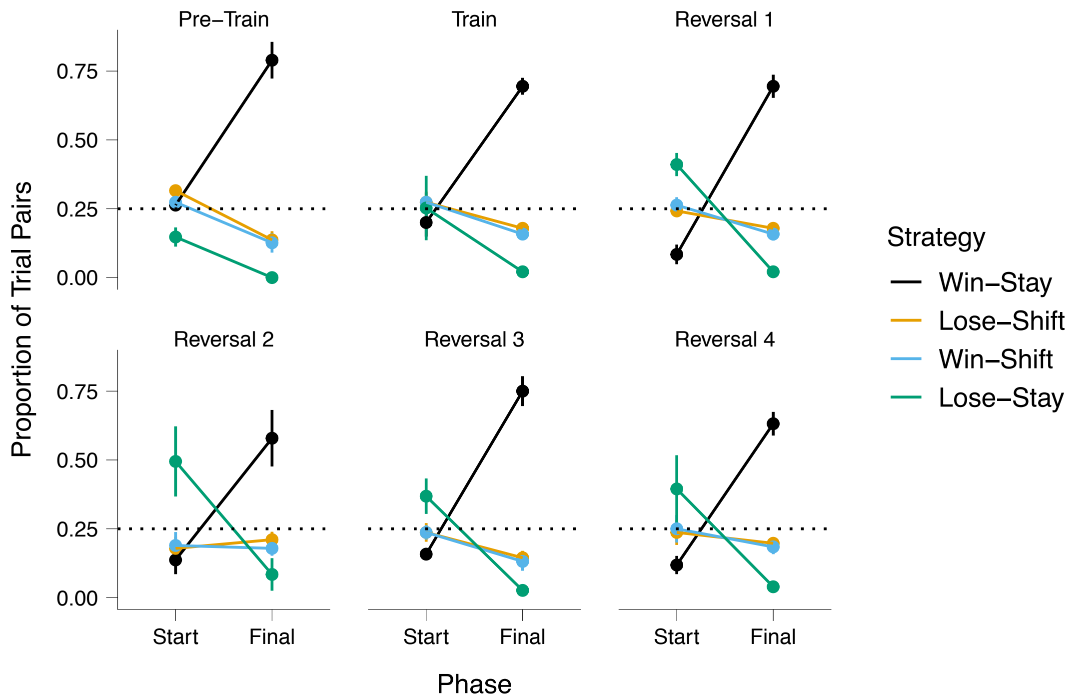

Figure 4. Observed proportions (mean ± se, across all tortoises) of choice strategies assessed from subsequent trial pairs, based on a win/lose-shift/stay categorisation for the first 20 trials (Start) and last 20 trials (Final) within each learning stage. Within 20 trials, each of the 19 pairs of trials were categorised as either win-stay, lose-shift, win-shift, or lose-stay based on whether the tortoise chose the positive stimulus (win) or neutral stimulus (lose). Random results would exhibit equal proportion (0.25; dotted line in figure) in each of these 4 categories.

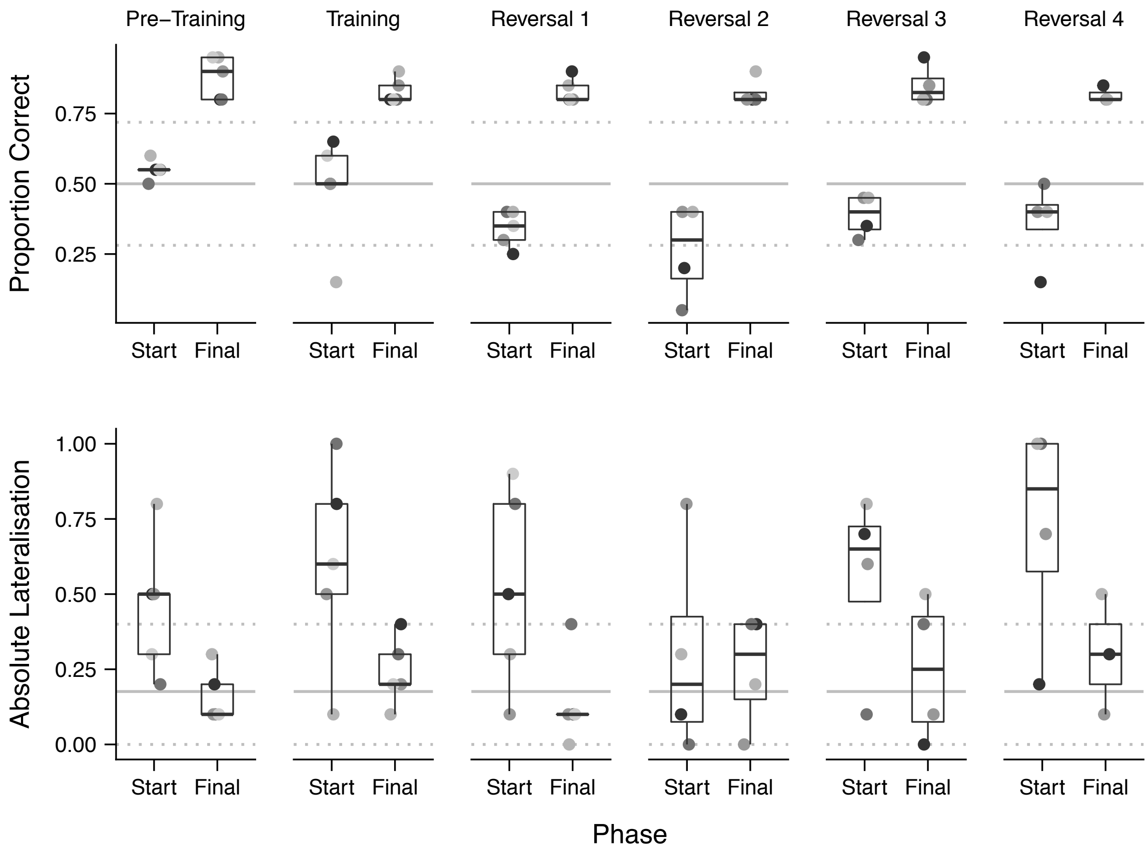

Figure 5. Learning performance and position bias across the different reversal learning paradigms in red-footed tortoises, expressed as box and whisker plots (median ± 25th and 50th percentile). The first 20 trials within each learning stage represent the starting condition, while the final 20 trials represent the phase where the learning criterion was reached. In the upper plots, the proportion correct represents the proportion out of 20 trials where the tortoise correctly selected the positive stimulus. In the lower plots, the absolute lateralisation refers to the side preference exhibited with respect to the dominant side chosen. Horizontal lines represent the mean ± 95% density regions for a binomial response with 20 samples and p=0.5, calculated by bootstrapping 10,000 times. Tortoises start out performing at random chance but often with a particular bias for a given direction and must lose this bias by the final 20 trials when the criterion is met.