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Artificial selection for reversal learning reveals limited repeatability and no heritability of cognitive flexibility in great tits (*Parus major)*

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Abstract

Cognitive flexibility controls how animals respond to changing environmental conditions. Individuals within species vary considerably in cognitive flexibility but the micro-evolutionary potential in animal populations remains enigmatic. One prerequisite for cognitive flexibility to be able to evolve is consistent and heritable among-individual variation. Here we determine the repeatability and heritability of cognitive flexibility among great tits (*Parus major*) by performing an artificial selection experiment on reversal learning performance using a spatial learning paradigm over three generations. We found low, yet significant, repeatability ($R = 0.15$) of reversal learning performance. Our artificial selection experiment showed no evidence for narrow-sense heritability of associative or reversal learning, while we confirmed the heritability of exploratory behaviour. We observed a phenotypic, but no genetic, correlation between associative and reversal learning, showing the importance of prior information on reversal learning. We found no correlation between cognitive and personality traits. Our findings emphasize that cognitive flexibility is a multifaceted trait that is affected by memory and prior experience, making it challenging to retrieve reliable values of temporal consistency and assess the contribution of additive genetic variation. Future studies need to identify what cognitive components underly variation in reversal learning and study their between-individual and additive genetic components.

Keywords: cognition, artificial selection, animal personality, quantitative genetics, reversal learning

Introduction

The need for animals to adjust their behaviour to environmental changes has selected for the evolution of cognitive abilities, that is, the abilities to acquire, process, store and act on information from the environment [1]. Animals show consistency during foraging [2,3] and are capable of memorizing various features of a food source, such as their location or shape [1,4]. However, the ability to re-evaluate this behaviour can be equally essential if, for example, a source no longer provides food. One particularly relevant trait, therefore, is cognitive flexibility, the executive cognitive function that allows animals to update goal-directed behaviour in response to changing environmental conditions [5]. Frequent fluctuations in ecological resources, such as in spatially complex and heterogeneous environments, should select for greater cognitive flexibility, allowing individuals to respond more rapidly to changes in their environment [1,6]. However, whether cognitive flexibility can respond to selection is currently unknown. To understand this evolutionary potential of cognitive flexibility, we need to quantify the extent of stable individual differences in cognitive flexibility and whether that individual variation has a genetic basis [7–9].

Determining stable individual differences in cognitive traits is crucial when investigating the adaptive nature of such traits, nevertheless, this has received relatively little attention in non-human animals [10], although the literature is expanding [11]. To test whether individual differences in performance are consistent over time, studies so far have focused on the existence of consistent among-individual variation, or *repeatability* of cognitive traits [12], finding that repeatability is usually below 0.28, with a significant publication bias in favour of reporting higher repeatabilities [11]. Cognitive flexibility is generally assessed with a reversal learning task [5]. In this assay, a previously rewarded option becomes non-rewarding and vice versa, in order to assess how flexible an animal can adapt its learned response by measuring the ability of animals to correctly respond to this change [13]. There is mixed evidence that reversal learning performance is repeatable: some studies report significant repeatability such as in great tits (*Parus major*) [14,15] and Australian magpies (*Cracticus tibicen dorsalis*) [16,17], but in blue tits (*Cyanistes caeruleus*) [14] and song sparrows (*Melospiza melodia*) reversal learning was not significantly repeatable [18]. In the latter case, the authors suggest the lack of

repeatability is due to a change in the nature of a task when it is measured repeatedly. Because of familiarity with the task, the performance might improve due to (rule-)learning or increased motivation [10,13]. They also argue that potentially the same performance measure assesses a different cognitive trait when the task is repeated [18]. Nonetheless, repeated measures of cognitive performance are needed to estimate consistent among-individual variation, in order to support the idea that there are individual differences in performance [10].

Repeatability estimates provide a fundamental upper limit for heritability, but consistency can also arise from early environmental or maternal effects [19]; but see [12]. Therefore, demonstrating that a phenotypic trait is heritable is a fundamental next step in understanding the trait's evolutionary potential [20]. Since reversal learning performance is always tested following an associative learning task, they are generally considered in concert. Assessing the extent of among-individual covariance between the two learning phases will be crucial to understand what drives, possibly heritable, among-individual differences in reversal learning performance. Following the hypothesized trade-off between learning and cognitive flexibility, animals that are quick at forming initial associations learn subsequent similar information less well [21–23]. Several studies have investigated both the phenotypic correlations between and the heritability of associative and reversal learning performance [24–31]. Current evidence suggests low to moderate heritability of reversal learning performance across species, but how it relates to associative learning appears to vary between species. Identifying the existence of a trade-off between learning and cognitive flexibility is important, because this has been hypothesised to maintain cognitive variation [22].

In line with this, personality traits and cognitive performance have been hypothesized to be correlated at the among-individual level [10]. Fast explorers would learn faster but inflexibly, meaning that they have more difficulty learning a new cue-reward association [32,33]. However, even though correlations have been found between personality traits and (reversal) learning performance, the direction of this relationship is unclear [32,34], and generally raw phenotypic correlations with no partitioning of among- and between-individual components are calculated. Knowing to what extent variation in personality traits relates to reversal learning performance in great tits, will help us

88 understand whether cognitive flexibility and personality variation are controlled by the same underlying
89 factors in great tits [35].

90 Experimental evidence for heritable variation in cognitive flexibility, or in any cognitive trait,
91 is lacking in non-human animals [36]. Artificial selection experiments provide a powerful method for
92 obtaining strong, direct evidence for naturally occurring, heritable individual variation in cognitive
93 performance [7]. Therefore, we here performed bi-directional artificial selection on natural variation in
94 spatial reversal learning ability under controlled laboratory conditions in hand-reared great tits (*Parus*
95 *major*) collected from a natural population, while including repeated measures to estimate consistent
96 among-individual differences. Previous artificial selection experiments in great tits have successfully
97 demonstrated heritability of exploratory behaviour, risk-taking behaviour and timing of reproduction
98 [37–39]. Here we estimated (i) individual consistency and repeatability of performance on associative
99 and reversal learning tasks, (ii) the covariation between performance on the associative and reversal
100 learning task and two personality traits, and (iii) the narrow-sense heritabilities of these cognitive traits.
101 We predicted significant, but low to moderate, individual consistency and heritability, since within-
102 individual variation in cognitive performance is likely influenced by environmental effects, experience,
103 and intrinsic motivation. We expected to find associations between associative learning, reversal
104 learning, and personality traits; negative associations would support the above suggested trade-offs,
105 while positive correlations could indicate they are determined by similar underlying mechanisms.

Methods

Study subjects

We conducted this study from May 2018 to April 2021 spanning 3 years. Each year in May, we hand-reared great tit (Parus major) nestlings until independence. As a starting population, we collected 322 10-day old nestlings from 42 broods originating from the nest box population Boslust (Groot Warnsborn) near Arnhem, the Netherlands (5°850 E, 52°010 N), a 70 ha field site consisting of mixed pine-deciduous forest. We brought them to the aviary facilities at the Netherlands Institute of Ecology (NIOO-KNAW) and hand-reared the nestlings until independence according to the methods described by [37]. Briefly, we transferred nestlings in sibling groups of three to four birds to a wooden box, each box containing three compartments with in each a natural parasite-free nest. Upon fledging, around 17-20 days after hatching, birds were transferred to small wire-mesh cages in groups of three. Around day 35 after hatching**,** they were completely independent and transferred to standard individual cages of 0.9 m × 0.4 m × 0.5 m with solid bottom, top, side, and rear walls, a wire-mesh front and three perches. Birds were kept under natural light conditions in acoustic and visual contact with each other.

After two days of individual housing, in June/July, we performed a novel environment test and a novel object test to test for early exploratory behaviour ([37]; further details below). In July, we took a blood sample for sex determination. In September, after their first moult, we transferred individuals to single-sex groups (maximum 7 males or 8 females) in semi-open outdoor aviaries (2 m x 4 m x 2.5 m), their standard housing outside the breeding season. Food consisted of a homemade mixture of ground beef heart, egg, calcium and a multivitamin solution, supplemented with mealworms, apple, and sunflower seeds and fat balls in winter, and was available ad libitum. In October, when birds were full-grown, we measured tarsus with sliding callipers to the nearest 0.1 mm. From October-February, birds were tested for associative and reversal learning performance to assess their cognitive flexibility. Based on their performance in the reversal learning task, we selected individuals for the 'reversal learning line' breeding pairs in the same semi-open aviaries to produce the next generation in captivity (for details see "*Selection procedure and breeding" below*).

Eggs produced by this parental (P) generation and the subsequent, first (F1) generation were placed in a natural nest at our field site to be incubated by wild foster females. Ten days after hatching, nestlings were collected, brought to our indoor facilities and hand-reared as described above (224 F1 chicks from 29 P pairs and 181 F2 chicks from 27 F1 pairs). These chicks were tested for exploratory behaviour for associative and reversal learning performance.

Reversal learning task

In total, we successfully tested 105 animals from 33 pairs (P), 149 (F1) and 131 (F2) individuals for reversal learning performance. From these birds, we tested 52 P, 55 F1 and 15 F2 individuals a second and 52 P individuals a third time to obtain repeated measures, from here on we refer to these repeated measures as 'testing rounds'. Supplementary Figure 1 and Supplementary Table 1 provide an overview of the sample sizes and timeline for the selection procedures as well as repeated measures.

Experimental apparatus

We used automated feeders to assess learning performance, using a three-choice spatial learning procedure. During each experiment, one feeder would provide a reward (freeze-dried mealworm) in a triangular array with two other unrewarding feeders, requiring individuals to learn the location of the rewarded feeder. A triangular array with three feeders is different from most setups of typically two choices. Spatial reversal learning paradigms involving additional choice options [14,15,23,40,41], make it possible to distinguish between error types and foraging strategies [13]. We used two feeder types for these experiments, the second type being a refined version that allowed for automated reversal switching.

Feeder type one (Supplementary Figure 2a): For the first testing round of the P generation, we used radio-frequency identification (RFID) controlled automated feeders [14]. Access to food (freeze-dried mealworms) was controlled by a transparent plastic door at the feeder opening, held in place by a solenoid. The solenoid would release for two seconds upon detection of a specific Passive Integrated Transponder (PIT) tag, allowing an individual access to one food item by pushing open the door. RFID readings and solenoid activation were controlled by a custom program loaded onto a printed circuit

board ('Darwin Board', Stickman Technologies Inc., UK). All visits and their timestamps were monitored with the RFID antenna throughout the experiment.

Feeder type two (Supplementary Figure 2b): In subsequent experiments (P generation testing rounds two and three, F1 and F2 all testing rounds), we substituted the above-described feeding stations for automated "smart" feeders, designed by NatureCounters (Maidstone, UK). These feeders functioned similarly to the Type one devices, and were also equipped with an RFID antenna. Opening and closure of a transparent plastic door was controlled with a micro-servomotor. Upon registration of a PIT-tag, the door opened for a set duration of two seconds. The three feeders were connected through a network cable, allowing for integrated responses between the feeders.

Training

Eight days prior to testing, individuals were trained to use the feeders in semi-open outdoor aviaries. Before training, birds were weighed and provided with a PIT-tag. During this training period, food and water were available ad libitum, but mealworms were excluded from the diet. On day one of the training period, we placed one or two feeders in the aviary, with the doors permanently open so that birds could learn to eat from the open door. On days two to seven, the door of the feeders was closed and set to a mode where it could only be opened when triggered by a successful transponder reading of any of the birds. On day eight, birds were selected for testing if during that week, they had > 80 visits per day at least once, > 30 visits per day at least twice (mean of maximum visits per bird on the day it had the most visits = 268 visits), see Supplementary Figure 3a for distribution) or >30 visits per hour at least once (mean of maximum visits per bird in the hour it had the most visits = 60 visits, Supplementary Figure 3b). Out of 546 trained individuals 54 did not pass this criterion. This selection criterion was chosen because the median number of visits that was needed to complete both the associative and reversal task was 31 visits. Hence, most individuals would be apt enough at using the devices to finalize the task within one day.

Learning experiment

We tested all birds between 9:00h and 15:00h. We caught individuals from the group, weighed them, and released them individually in a testing aviary. In this testing aviary, water was available ad libitum and food was not available to maintain the motivation to take part in the learning test. Testing took place with three feeders positioned in a triangular array, doors facing inwards.

In the associative learning phase, we *a priori* randomly chose the rewarded feeder. This assigned feeder was reinforced until individuals reached a criterion level of six correct trials out of seven. This success rate is significantly different from the expectation if birds selected feeders at random (binomial test, $p < 0.01$). A trial was defined as a landing on the perch.

After completing the associative learning phase, the reward contingencies were reversed to one of the other two feeders for the reversal learning task, which was randomly chosen *a priori*. All experimental tests were observed live through a one-way screen and upon reaching criterion, the observer initiated the reversal manually. Again, birds had to reach the learning criterion of six correct visits over seven trials to the new feeder. Both phases were filmed using a GoPro Hero5 (GoPro inc.) placed inside the aviary in case rewatching was necessary. After the test, birds were caught, weighed and released back in their home aviary. Associative and reversal learning performance were scored as the total number of trials required to reach the learning criterion, including those last seven visits.

After testing the parental generation with the first feeder type, we refined our testing feeder to allow for automated high-throughput phenotyping. The following changes were implemented for feeder type two: (i) For the associative learning phase, the first feeder that a bird visited was rewarded, instead of a random feeder. This ensured that any variation in reversal learning performance would not be due to variation in preference. (ii) The devices were programmed such that the rewarded feeder switched automatically to initiate the reversal phase when the learning criterion was reached. Birds continued serial reversals until caught from the aviary at 15:00. For all analysis in this paper we only included the first reversal of each testing round. (iii) Due to a difference in design, the doors faced away from the centre of the three devices, instead of inwards. (iv) Energetic state and satiation can affect evaluation of the food reward as well as learning speed [42]. Therefore, we offered dry egg food ad libitum in the test aviary during the experiment. Great tits strongly prefer the dried mealworm rewards in the feeders over

dried egg food. Despite these differences, the basic principles of the test remained the same. In all analyses, we controlled for the type of feeder used to measure learning performance.

Exploratory behaviour and boldness

To test birds for exploratory behaviour, we conducted a novel environment test in the observation room after two days of individual housing (for more details, see [37,43]) We used the total number of flights (movements between trees) and hops (movements between branches within trees) within the first two minutes as 'exploratory score' [44]. We measured the response to novel objects on day one and day two after the novel environment test ($R = 0.54$, $CI = [0.4,0.6]$, $p < 0.001$). We measured the latency to approach the object and the shortest distance to it within 120 s, and the results for each test were converted linearly to a 0 – 5 scale. A score of five was given when the bird pecked the object, a score of zero when the bird did not land on the perch on which the object was situated within 120 s. A score of 1-4 is given depending on the time it took a bird to land on the perch and the minimal distance to the novel object. We summed scores from both novel object tests as an index of boldness (0 – 10, 'boldness score').

Selection procedure and breeding

From the parental generation, individuals were selected to breed based on their reversal learning performance in the first testing round. Individuals were ranked on the number of trials they required to reach learning criterion. For both the 'fast' and 'slow' learning lines we selected 18 males and 18 females and created 'fast' and 'slow' pairs. For each sex, we included a maximum of three siblings per line. Within each line, we ranked the selected birds for reversal learning performance for the two sexes separately. We then paired males and females that had contrasting reversal learning performances within each line (e.g. for the fast line a relatively fast performing fast male with a relatively slow performing fast female), avoiding full-sib mating. In this way we avoided creating most variation between broods within a line rather than between lines. Mid-parent values and performance ranges for the pairs that produced offspring that was subsequently successfully tested are reported in Supplementary Table 2.

From February onwards, breeding pairs were housed in semi-open outdoor aviaries (approximately 2.0 m x 4.0 m x 2.5 m) under natural photoperiod and temperatures. Each aviary contained four nest boxes. From March onwards, daily additional light was supplied from a single full-spectrum daylight fluorescent lamp (58W, 5500K, Truelight, the Netherlands) per aviary. Lights were on from 2.5 hr before sunrise (but never earlier than 02:00) until 24:00 to synchronize their breeding with the wild population. Moss and dog hair was supplied from mid-March onwards as nesting material.

Nest boxes in the aviaries were checked twice a week for nest building. When nests were complete, next boxes were checked daily for egg laying. Freshly laid eggs were collected, replaced with dummy eggs, and stored for a maximum of 14 days in an egg-turner. After five days of incubation, the nests and the dummy eggs were removed from the nest box and females were allowed to relay. All eggs incubated by wild foster females (~14 days) at the nest box population in Boslust. For the F1 generation, in order to increase hatching success in the wild, about one-third of broods was first incubated by the genetic mother for five days in the aviary and then placed in a wild nest to be further incubated by foster females (additional ~9 days). Around 50% of clutches was fostered as one unit, the other 50% was split up across two or more foster nests.

To produce the F2 generation, we also formed 18 pairs for each line from the F1 generation as described above for the P generation. Not all pairings bred successfully. We obtained 86 chicks from 16 'fast' broods and 63 chicks from 12 'slow' broods from the P generation, and 37 chicks from 10 'fast' broods and 94 chicks from 16 'slow' broods from the F1 generation (Supplementary Figure 1).

Statistical analysis

For both associative and reversal learning performance, we used the number of trials to reach learning criterion as performance measure. Across 56 out of 573 reversal learning tests, the criterion was not reached before 15:00 PM, meaning trials to criterion was censored. To investigate if performance differed between associative and reversal learning while including censored individuals, we compared survival curves between the associative and reversal learning task. We used the functions *Surv* and *Survfit* from the 'survival' package [45] to compute Kaplan-Meier survival curves. We employed the

Kaplan-Meier method to estimate number of trials to criterion for censored individuals. We used an individuals' maximum number of trials for "follow up time", and success (0 = criterion not reached, 1 = criterion reached) for "event". We used the function *survdiff* [45] to compare the two survival curves of the associative and reversal learning task, by computing a log-rank test employing a chi-square test statistic. As expected, individuals needed more trials to finish the reversal learning task compared to the associative learning task (log-rank: χ^2_1 =136, $p < 0.001$, Supplementary Figure 4).

To include censored data in the linear regression models to calculate heritability and repeatability, we computed so-called pseudo-values of the restricted mean trials to criterion from the Kaplan-Meier survival curves [46]. As a measure of trials to criterion, pseudo-values were computed for all observed trials, both censored and uncensored, using the function *pseudo* [45]. Pseudo-values were evaluated at the maximum trial number reached by the individual with the highest trials to criterion. How the values for trials to criterion changed from raw to the pseudo values is illustrated in supplementary Figure 5. To prevent confusion, the pseudo-values will hereafter be referred to as 'trials to criterion (TTC)'.

To test whether variation in associative and reversal learning performance can be explained by feeder type, test round, start time, body condition (residuals of the regression of body weight at start of the experiment against tarsus) or sex, we constructed linear mixed models with the function *lmer* in the 'lme4' package [47] with these explanatory variables and trials to criterion (ln-transformed) as the dependent variable. To account for multiple observations, animal ID was included as random intercept. We created separate models for associative and for reversal learning performance. Significance of fixed-effects was tested using Type II ANOVAs Kenward-Roger degrees of freedom with the function *Anova* in the 'car' package [48]. We evaluated terms using F-tests.

Heritability and repeatability of associative and reversal learning performance

We used two approaches to assess narrow-sense heritability of associative and reversal learning. Narrow-sense heritability (h^2) is the proportion of total phenotypic variance (V_P) that is attributed to the additive effect of genes, i.e., additive genetic variance (V_A) [19]:

$$h^2 = \frac{V_A}{V_P} \quad (1)$$

First, we estimated narrow-sense heritability of reversal learning performance using the response to artificial selection on performance on the first testing round. Because we only tested the parental generation with the first feeder type, we could not control for this using only data from the first testing round. Therefore, we used reversal learning performance corrected for feeder type, by using the residuals from the following model as response variable: a linear model of the complete dataset including all testing rounds (in later testing rounds, the parental generation was tested with feeder type two), with trials to criterion (ln-transformed) as the dependent variable, and feeder type as the fixed effect. Estimates of heritability based on the response to selection are referred to as realized heritabilities [19], and can be estimated from the breeder's equation,

$$R = h^2 S \quad (2)$$

By calculating the ratio of observed response to selection (R), the difference between mean offspring value and mean parent value, to observed selection differential (S), the difference between mean value of selected parents (weighted for number of tested offspring) and mean parent value, we estimated the realized heritability:

$$\hat{h}_r^2 = \frac{R}{S} \quad (3)$$

To estimate realized heritability over several generations of selection, we summed R and S over successive generations respectively, to acquire the cumulative selection response $R_C(t)$ and cumulative selection differential $S_C(t)$. Then, by regressing $R_C(t)$ on $S_C(t)$, we estimated realized heritability as the slope of this regression [19]. This approach allowed us to show the selection differentials in relation to the response to selection in our experiment. However, it also returns a larger error around the estimates and does not allow for further variance partitioning. Therefore, we additionally estimated the heritability, as well as the repeatability of associative and reversal learning performance, by fitting univariate Bayesian mixed-effect animal models with the 'MCMCglmm' package [49]. These models included the following random effects: individual identity to account for repeated measures and estimate permanent environment effect (V_{PE}), individual identity linked to the pedigree to estimate additive genetic variance (V_A) and maternal identity to estimate maternal variance (V_M). In addition, feeder type

323 was included as fixed effect. For these univariate models, we used the complete data set including all
324 repeated measures. For both associative and reversal learning performance, we used trials to criterion as
325 pseudo values rounded to the nearest integer as response variable.

326 Estimates of genetic variance (V_A), were extracted from the full animal model and scaled by the
327 total phenotypic variance (V_P, calculated as the sum of the estimated variance components and V_R
328 representing within-individual variance originating from plasticity and/or measurement error). As such,
329 heritability (h^2), was estimated as:

330 $$h^2 = \frac{V_A}{V_P} = \frac{V_A}{V_A + V_M + V_{PE} + V_R} \quad (4)$$

331 Repeatability was calculated as the proportion of phenotypic variance explained by the individual [19],
332 with V_I representing among-individual variance (V_A+V_{PE}+V_M) and was estimated as:

333 $$R = \frac{V_I}{V_P} = \frac{V_A + V_M + V_{PE}}{V_A + V_M + V_{PE} + V_R} \quad (5)$$

334 For personality, response variables were either 'exploratory score' or 'boldness score'. We had no
335 repeated measures, so estimated only additive genetic variance (V_A) and maternal variance (V_M). The
336 estimate of genetic variance was extracted from the animal model and scaled by the phenotypic variance
337 to yield estimated heritability (h^2).

338 We modelled all four latent traits using a Poisson error distribution, and therefore used a
339 parameter expanded prior suitable for univariate poisson models (Fisher prior, V = 1, nu, = 1, alpha.mu
340 = 0, alpha.V = 1000) that is more informative toward the expected small variances, and puts less weight
341 in small values close to zero [50]. We present the estimates as posterior means with the associated 95%
342 CI on the latent scale and also converted these estimates to the observed scale (R_{obs} using the *QGicc*
343 function, h^2_{obs} using the *QGparams* function, from the "QGglmm" R package [51]). Since variance
344 components are constrained to be positive in MCMCglmm, we used closeness of the lower bound of the
345 credible interval to zero to estimate confidence in a non-zero proportion of the variance components.
346 For fixed effects, differences were considered significant when 95% credible intervals (CI) for effect
347 sizes did not overlap with zero.
348

Pairwise correlations between associative learning performance, reversal learning performance, exploratory behaviour and boldness

To estimate genetic and non-genetic covariance between associative and reversal learning performance, we fitted two bivariate animal models using the complete data set including all repeated measures: one with feeder type as a fixed effect, and a second model where we partitioned the variance-covariance at the two feeder type levels. These models both included individual identity (V_{PE}) and individual identity linked to the pedigree (V_A) as random effects. We left out maternal identity (V_M) from the second model to avoid overfitting and because maternal effect was estimated to be near zero for both traits. To estimate covariance between the learning and personality traits, we fitted bivariate animal models. Because of the lack of repeated measures for the personality traits, we included only performance on the first test round for the learning traits. These models included individual identity linked to the pedigree (V_A) as random effect.

We used a Poisson error distribution, and a parameter expanded prior suitable for bivariate poisson models that is weakly informative for the small variances expected for the Poisson (V = diag(2), nu, = 2, alpha.mu = c(0,0), alpha.V = diag(2)) [50]. We calculated the phenotypic, genetic, permanent environment and/or maternal correlations by dividing the covariance between each two traits by the product of the square root of their variances, e.g.:

$$Genetic\ correlation = \frac{\text{additive genetic covariance}}{\sqrt{(V_{A,Trait1} * V_{A,Trait2})}} \quad (6)$$

We used the 95% CI to assess statistical significance of the covariances, which were considered significant when not overlapping with zero. We ran each model for a minimum of 100,000 iterations, with a burn-in of 10,000 and a 10- or 50-iteration thinning interval, to obtain sample sizes >1000 (with some exceptions >400) and autocorrelation <0.20 (with some exceptions <0.50). Convergence of MCMC sampling and autocorrelation of chains were assessed visually following [50].

To visualize the relationship between associative and reversal learning performance, we employed a linear model using function *lm* from the 'stats' package [52], with trials to criterion of reversal learning performance (ln-transformed) as the dependent variable and trials to criterion of associative learning performance (ln-transformed), feeder type and their interaction as fixed

effects, and used the function *interact_plot* from the 'jtools' package to plot the significant interaction. Additionally, for feeder type one, where we had randomly assigned the rewarded feeder a priori, we additionally explored how the relationship between reversal learning performance and associative learning performance depended on the number of trials it took a bird until the first correct visit was made, which we called 'correct visit lag number'. If the correct visit lag number equalled 1, the first feeder that an individual chose to land was also the rewarded feeder. If the correct lag visit number equalled 2 or higher, the individual first visited other unrewarded feeders. A higher correct visit lag number might indicate slow learning or a strong tendency to visit the other unrewarded feeder(s). For this, we used a linear model using function *lm* with trials to criterion (ln-transformed) of the reversal learning phase as the dependent variable, and trials to criterion (ln-transformed) of the associative phase, 'correct visit lag number' and their interaction as fixed effects. Significance of fixed- effects was tested using Type III ANOVAs and Kenward-Roger degrees of freedom. We evaluated terms using F-tests. Post hoc comparisons were performed with the function *emtrends* command in 'emmeans' package [53] to compare estimates of slopes of the trials to criterion trend for each level of feeder type and mean ± SD of 'correct visit lag number', using the Tukey test to correct for multiple comparisons. We used the function *interact_plot* from the 'jtools' package to visualize the interaction.

All analyses were conducted in R version 4.0.3 [52].

Results

Heritability and repeatability of associative and reversal learning performance

The significance and magnitude of fixed effects varied across task performances. These effects are not directly relevant to hypotheses being tested but are reported in full in Supplementary Table 3. Feeder type had a significant effect and was therefore controlled for all in subsequent models. The level of repeatability (with 95% credible interval (CI)) of associative learning performance was 0.105 (0.015, 0.198) on the latent and 0.065 (0.011, 0.128) on the observed data scale (Table 1). For reversal learning, the level of repeatability was 0.153 (0.038, 0.287) on the latent and 0.116 (0.028, 0.220) on the observed data scale (Table 1). There was no support for heritable variation in associative and reversal learning performance in our selection experiment with estimates of narrow-sense heritability of 0.020 (0.000,0.074) on the latent and 0.012 (0.000, 0.044) on the observed data scale for associative learning performance, and 0.030 (0.000, 0.099) on the latent and 0.022 (0.000, 0.072) on the observed data scale for reversal learning performance and the 95% CI for these h^2 values including zero. On the contrary, estimates of narrow-sense heritability for exploratory behaviour were 0.499 (0.239, 0.766) and 0.265 (0.114, 0.399) on the latent and observed scale, and 0.463 (0.175, 0.714) and 0.252 (0.110, 0.410) on the latent and observed scale for boldness. In all cases, permanent-environment effects and maternal effects explained less than 5% of the phenotypic variance and the lower limit of the 95% CI was zero (Table 1).

Realized heritability of reversal learning performance

Great tits from the 'fast' and 'slow' line did not differ in reversal learning performance after one (t-test: $t_{151} = 0.46$, $p = 0.65$) and two (t-test: $t_{129} = -0.89$, $p = 0.37$) generations of selection. The realized heritability (response to selection as proportion to selection differential) was 0.02 ± 0.03 and did not differ significantly from zero (Linear regression: $F_{1,4} = 0.55$, $P = 0.50$, $R^2 = 0.12$, Figure 1).

Table 1 *Estimates of fixed effects, variance components, repeatability and heritability of cognitive and personality traits in the great tit, obtained from univariate animal models. Repeatability and heritability are given on the latent scale (R_{lat}, h^2_{lat}) as well as on the observed scale (R_{obs}, h^2_{obs}). Estimates are given as posterior mean with 95% credible intervals. Significant estimates (95% CI does not include zero) are in bold.*

Variable	Associative learning Posterior mean [CI]	Reversal learning Posterior mean [CI]	Exploratory behaviour Posterior mean [CI]	Boldness Posterior mean [CI]
Fixed effects				
Intercept	2.989 [2.833,3.122]	3.369 [3.246,3.498]	0.909 [0.731,1.132]	1.034 [0.829,1.226]
Feeder Type (2)	-0.603 [-0.755,-0.441]	-0.288 [-0.416,-0.146]		
Variance components				
Additive genetic variance, V_A	0.010 [0.000,0.036]	0.011 [0.000,0.037]	0.304 [0.116,0.521]	0.263 [0.080,0.460]
Residual variance, V_R	0.420 [0.355,0.487]	0.302 [0.241,0.356]	0.269 [0.137,0.406]	0.278 [0.141,0.430]
Permanent environment effect variance, V_{PE}	0.018 [0.000,0.059]	0.031 [0.000,0.076]		
Maternal effect variance, V_{MA}	0.022 [0.000,0.053]	0.013 [0.000,0.036]	0.030 [0.000,0.096]	0.022 [0.000,0.074]
Variance ratio				
R_{lat}	0.105 [0.015,0.198]	0.153 [0.038,0.287]		
R_{obs}	0.065 [0.011,0.128]	0.116 [0.028,0.220]		
h^2_{lat}	0.020 [0.000,0.074]	0.030 [0.000,0.099]	0.499 [0.239,0.766]	0.463 [0.175,0.714]
h^2_{obs}	0.012 [0.000,0.044]	0.022 [0.000,0.072]	0.265 [0.114,0.399]	0.252 [0.110,0.410]

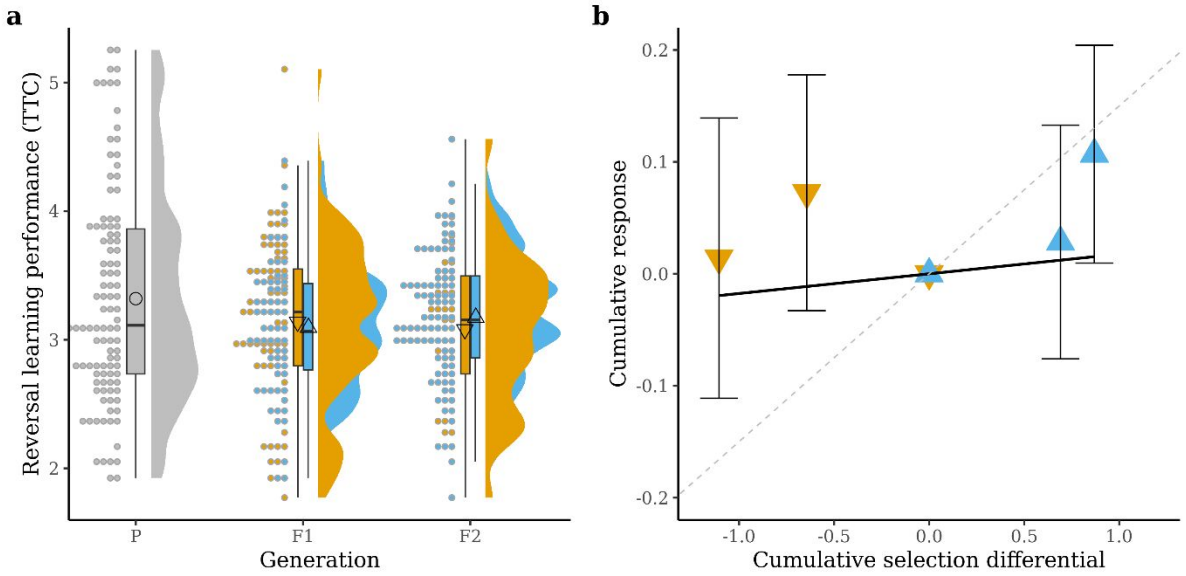

Figure 1 Response to selection per generation. *(a) Boxplot with horizontal lines representing median and interquartile range of trials to criterion, circle and triangles indicate mean trials to criterion, corrected for feeder type. Eye plots represent density distributions for trials to criterion. Dots indicate number of individuals within each bin. (b) Realized heritability (h^2) of reversal learning performance in an artificial selection experiment. Cumulative response to selection (R) is plotted as function of the cumulative selection differential (S) for each line selected on number of trials to reach learning criterion for two generations. The realized heritability (h^2) was calculated as the slope of the linear regression between R and S (black solid line). Error bars represent standard errors. Dashed line indicates expected function if slope (h^2) were 0.15. Yellow, downward pointing arrows: fast line; blue, upward pointing arrows: slow line.*

Covariances among associative learning performance, reversal learning performance and personality traits

We found a negative phenotypic correlation between associative and reversal learning performance, indicating that individuals that were slow during associative learning showed fast reversal learning. This phenotypic correlation is feeder type specific, with a significant negative correlation for feeder type 2, and a non-significant positive correlation for feeder type 1 (Figure 2a, Table 2). This correlation could not be explained at the genetic, permanent environment or maternal level, since these estimates were low with relatively large 95% confidence intervals (but note that the permanent environment correlation is similar to the phenotypic correlation; Table 3). We found no credible correlations between associative and reversal learning performance and the personality traits, neither at the phenotypic nor the genetic level (Table 2).

Table 2 Results from bivariate linear mixed models. *Phenotypic, genetic, permanent environment and/or maternal correlations between each pair of traits. All correlations are given as posterior means with 95% credible intervals. Significant correlations are in bold.Type 1 and 2 refer to feeder types 1 and 2.*

Traits	Phenotypic correlation	Genetic correlation	Permanent environment correlation	Maternal correlation
Associative learning /Reversal learning	**-0.097 [-0.190,-0.007]**	-0.003 [-0.485,0.507]	-0.022 [-0.865,0.828]	-0.074 [-0.914,0.802]
Associative learning /Reversal learning (Type 1)	0.113 [-0.090,0.304]	-0.013 [-0.707,0.702]	0.057 [-0.816,0.926]	
Associative learning /Reversal learning (Type 2)	**-0.201[-0.303,-0.089]**	-0.016[-0.515,0.492]	-0.213 [-1.000,0.730]	
Associative learning/Boldness	0.096 [-0.023,0.231]	-0.022 [-0.418,0.344]		
Associative learning/Exploration	-0.045 [-0.184,0.089]	-0.217 [-0.681,0.384]		
Reversal learning/Boldness	-0.124 [-0.250,0.010]	-0.120 [-0.672,0.579]		
Reversal learning/Exploration	-0.124 [-0.250,0.010]	-0.120 [-0.672,0.579]		

Using data from feeder type 1, we explored whether the fact that an individual needed to learn that a preferred or a random feeder was rewarded could explain the difference in direction of the relationship between associative and reversal learning. Indeed, there was a trend that the effect of associative learning on reversal learning performance depended on the latency to the first correct visit that was made during associative learning (LM; TTC associative * Correct visit lag number: $F_{3,100}$= 3.58, p = 0.06, Table 3). Post hoc comparisons show a significant positive relationship between associative learning and reversal learning performance when the 'correct visit lag number' is high (not learning initial choice, at mean +SD 0.5 ± 0.21 t_{100} = 2.30, p = 0.02), but that the slope is less steep when this number decreases (learning initial choice, at mean -SD –0.11 ± 0.18 t_{100} = -0.63, p = 0.52) (Figure 2b).

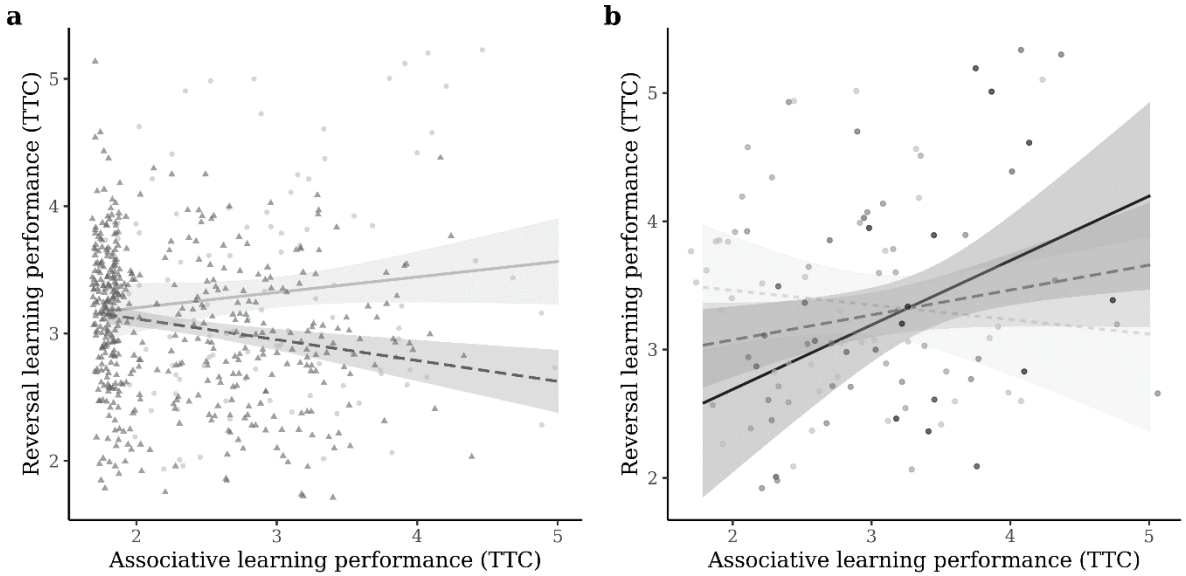

Figure 2 Relationship between the number of trials to criterion (ln-transformed) in the reversal learning phase and the number of trials to criterion (ln-transformed) in the associative learning phase. *Higher values = slower learning. This is shown for a) feeder type 1 (Light grey, continuous line and dots) and type 2 (dark grey, dashed line and triangles) and for b) the interaction between associative learning and the 'correct visit lag number' (grouping levels based on standard deviation; +1 SD is dark grey solid line and -1 SD is light grey dashed line). Darker dots reflect a higher 'correct visit lag number'. For both a) and b), plotted lines are marginal effects of the interactions term. Lines and shaded regions represent the model prediction (± 95 % confidence interval). The points of the scatterplot represent the actual data points.*

Table 3 *Associative learning performance in the first testing round as predictor of reversal learning performance for only feeder type 1 (n = 104), fitted with a linear mixed effect model with 'correct visit lag number' as fixed effect.*

Variable	Estimate	SE	F-statistic	p-value
(Intercept)	3.79	0.59		
TTC associative	-0.14	0.19	$F_{1,100} = 0.56$	0.46
Correct visit lag number	-0.37	0.20	$F_{1,100} = 3.33$	**0.07**
TTC associative * Correct visit lag number	0.11	0.06	$F_{1,100} = 3.59$	**0.06**

Discussion

To investigate the evolutionary potential of cognitive flexibility, we here estimated the among-individual variation and additive genetic variation of reversal learning and related traits using a bi-directional selection experiment of reversal learning performance in the great tit (*Parus major*). We found significant, but low, repeatability of reversal learning performance, but no evidence for narrow-sense heritability of reversal learning performance. We argue that this suggests a relatively low evolutionary potential of cognitive flexibility, but also points to difficulties with obtaining measures of cognitive flexibility.

The fact that reversal learning performance has a significant but relatively low repeatable component (R_{lat} = 0.153; 95% CI = 0.038,0.287), shows that performance on this task is to some extent

consistent over time. Because we used relatively long measurement intervals (mean = 345 days), our repeatability estimates may be somewhat conservative. Over longer time periods, the environment of an individual is more likely to change relative to the last measurement, increasing within-individual variation in performance [17]. Our findings are in line with other studies finding low repeatability of reversal learning performance in great tits [11,14,15]. An important reason for this low repeatability is the challenging nature of measuring cognitive traits accurately in general. Because individuals need to be trained in taking rewards before being tested, memory, motivation and prior experience are likely confounding effects on the performance on subsequent cognitive tests [10,18]. This is especially true for reversal learning tasks, because subsequent reversals induce a change in prior knowledge, essentially changing the trait that is measured [13]. Our results show that slight differences in the test setup between test rounds already affected the correlation between prior experience and reversal learning performance. This prior experience may be driven by chance effects, such as the first feeder on which a bird lands. In the future, a better understanding of the role of prior experience and memory needs to be identified. But also, knowledge on the reasons why there is a link between performance in the associative phase and the reversal phase is important in order to explain the large within-individual variance in reversal learning over consecutive trials.

Reversal learning performance did not respond to artificial selection over generations, and despite the low but significant repeatability, we found no evidence for heritable variation. The fact that, in line with previous research, we found evidence for heritability of both personality traits we measured, boldness towards a novel object and exploratory behaviour, shows that the power of our pedigree was sufficient to pick up moderate heritabilities [37,54,55]. The low heritability estimates and lack of response to selection as we found here, suggest that reversal learning performance has little to no evolutionary potential. Potentially, genetic variation may have been selected against for cognitive flexibility, since traits closely linked to fitness are expected to have little additive genetic variance, possibly lost through strong directional selection [8]. However, considering the broad phenotypic variation in cognitive flexibility in our study, that does not seem to be the explanation for the results in this study. Instead, considering the high amount of residual variance, there is still a great deal of

individual variation unaccounted for, and we speculate on some possible sources of variation in the discussion below.

Other studies did find moderate heritability in reversal learning performance, in red junglefowl (*Gallus gallus*) and house mice (*Mus musculus*), [24–26], although no heritability was found in Aegean wall lizards (*Podarcis erhardii*) [30]. What can explain the different findings among studies? In other work on the great tit, problem solving was shown to be a moderately repeatable composite behaviour [56] but not heritable [57]. Furthermore, repeatability in problem solving was shown to be explained entirely by the underlying behaviours (motivation, experience, selective attention; [58]), possibly explaining the lack of heritability in problem solving performance. Similarly, reversal learning performance is a composite trait made up of different cognitive components [59]; its heritability is therefore dependent on the additive genetic variation of each of these components, and on their covariance (as detailed below). We summarized reversal learning performance as the number of trials to reach a learning criterion. This is a commonly used performance measure, but it does not allow to disentangle the different cognitive components that determine performance. If different task types, experimental setups, or species, lean more towards usage of one of the components, our chosen performance measure, trials to criterion, might be the cause of the differences between our and other studies. Furthermore, since each cognitive component itself may also be influenced by environmental variation, total additive genetic variation on a summary measure like trials to criterion may be masked.

Reversal learning performance tests a combination of cognitive components, that trials to criterion may not adequately capture [60] such as: (1) the degree of proactive interference, where previously learnt, but now irrelevant, information interferes with learning and remembering newly relevant information [23,61]. Proactive interference results in perseverative choices and explained a large part of the among-individual variation in reversal learning performance in great tits [15]; (2) attentional allocation, the ability to notice that the change has occurred, resulting in a potential failure to detect task transitions [13]. For example, high resistance against external sources of interference (external attention) impairs reversal learning performance [62]; and (3) learning the new cue-reward association through reinforcement learning [15]. Reinforcement learning is involved in both associative and reversal learning performance since both phases require individuals to learn from positive

reinforcements, increasing the excitatory strength of a stimulus, and to learn from non-reinforcements, decreasing approach towards non-rewarded locations [59]. Sensitivity to negative as well as to positive feedback are stable traits [63]. Each of these constituents may be separate components of reversal learning performance that selection can act upon, but the extent to which these components make up reversal learning and the heritabilities of these traits remain an important avenue of further study.

These different cognitive components are also important for understanding our findings on the relationships between associative learning and reversal learning performance. The relative importance of proactive interference suggests that reversal learning performance can be strongly dependent upon the outcome of the associative learning task. We found that associative and reversal learning performance are negatively correlated, in line with a hypothesized trade-off between learning and cognitive flexibility, in which animals that are quick at forming initial associations learn subsequent similar information less well [21–23]. However, we only observed this negative correlation when individuals were rewarded for their first choice during the associative learning task, which may be their preferred feeder. Individuals that were quick to learn their initial choice possibly had a stronger preference for this feeder, developed a stronger memory, and experienced more proactive interference during the reversal task. Strong preferences are difficult to reverse [64], and faster and stronger associative learning might make it more difficult to inhibit the response towards that cue [65,66]. On the other hand, when a randomly allocated feeder was rewarded first, there was a trend for a positive correlation between associative learning and reversal learning performance, if individuals were forced to learn a non-preferred feeder during the associative learning phase. Perhaps a stronger memory is formed if an individual takes a long time to learn a feeder to which it does not have a strong initial tendency to visit, causing more proactive interference of that memory, explaining the positive correlation between associative and reversal learning performance [27].

Following the speed-accuracy trade-off hypothesis [33], we predicted that more exploratory and bolder individuals, who are supposedly less sensitive to new information, would therefore be worse at reversal learning performance while being better at associative learning tasks. However, contrary to other studies (Guillette *et al.*, 2011; Marchetti and Drent, 2000; Mazza *et al.*, 2018; van Oers, Klunder and Drent, 2005; but see Guillette *et al.*, 2015), our findings do not support a speed-accuracy trade-off

as we find no evidence for either phenotypic of genetic covariance between learning and personality traits. Perhaps this relationship cannot be identified at the resolution of the performance criterion we used here. For example, bolder sticklebacks performed better during associative learning and made more mistakes during reversal learning, but only during the first few trials, whereas these differences were not visible at trials to criterion [72]. This again suggests that dissecting the different mechanisms underlying reversal learning performance and relating them to associative learning and personality traits will be a crucial step towards a better understanding of any trade-offs between learning, flexibility and personality.

In summary, our results demonstrate that reversal learning performance is somewhat repeatable over longer time periods but seems to have no additive genetic variation. Although this suggests a limited evolutionary potential, the intricate relationships with associative learning performance suggests a more complex interplay of experiences that results in task performance, possibly explaining the lack of additive genetic variation here. For future studies, it will be crucial to disentangle the different components that likely underlie individual variation in reversal learning performance; reinforcement learning, proactive interference, and attentional allocation. This will allow for separately quantifying the repeatability and heritability of these components as well as their covariances, and their relationships with other behavioural traits and ultimately fitness. This should preferably be done across a range of environmental conditions to explore the possibility that selection acts on the level of reaction norms rather than on the reversal learning performance itself.

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