Impulsivity is affected by cognitive enrichment and links to brain gene expression in red junglefowl chicks

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Despite reported findings, explanations for within-species variation in behavioural performance on cognitive tests are still understudied. Cognitive processes are influenced by environmental and genetic differences, where cognitive stimulation and monoaminergic systems are predicted to be important. To explore explanations for individual variation in impulsivity (a behaviour that is negatively correlated with inhibitory control), we experimentally altered the environment of red junglefowl, Gallus gallus, by exposing chicks from newly hatched to 9 weeks old to either (1) both environmental and cognitive enrichment, (2) environmental enrichment without additional cognitive enrichment or (3) neither environmental nor cognitive enrichment. Subsequently, we measured variation in impulsivity and brain gene expression of genes from the dopaminergic system (DRD1 and DRD2) and serotonergic system (SH2A, SHT1B, SHT2B, SHT2C and TPH). We focused on two aspects of impulsivity, impulsive action and persistence, and their reduction over time. Cognitively enriched chicks tended to have higher initial impulsive action and had higher initial persistence, and our environmentally enriched chicks had slower reduction of impulsive action over time. DRD2 (a dopamine receptor gene) had lower expression in environmentally enriched chicks. Variation in impulsive action tended to correlate with expression of TPH (a gene involved in serotonin synthesis), whereas persistence correlated with both TPH and the dopamine receptor gene DRD1, and tended to correlate with the dopaminergic gene DRD2, regardless of rearing treatment. These results indicate that both environment and links to neurobiology could explain individual initial variation in, and reduction of, impulsivity. Further, distinct neurobiological pathways appear to govern impulsive action versus persistence, supporting the suggestion that impulsivity is a heterogenic behaviour.

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Individual variation in behavioural performance on cognitive tests is repeatedly observed across taxa (e.g. Bensky & Bell, 2018; Kopplik, Hoffmeister, Brunkhorst, Kieß, & Thié, 2015). This implies that individuals within species can differ in cognition (i.e. how they perceive, store and act on information from environmental stimuli; Shuttleworth, 2010). However, explanations to account for such variation remain unclear for many cognitive traits (Boogert, Madden, Morand-Ferrat, & Thornton, 2018; Shaw & Schmelz, 2017; Thornton & Lukas, 2012).

Inhibitory control (i.e. the ability to inhibit one response in favour of another, Brucks, Marshall-Pescini, Wallis, Huber, & Range, 2017; Logan, Schachar, & Tannock, 1997; Shaw, 2017) is a cognitive trait important for avoiding behavioural responses that could be counterproductive or maladaptive in both social and asocial situations (e.g. Amici, Aureli, & Call, 2018; Boogert, Anderson, Peters, Searcy, & Nowicki, 2011). Inhibitory control is negatively correlated with impulsive behaviour (i.e. reactions to stimuli that appear to occur without complete processing of the available information; Moeller, Barratt, Dougherty, Schmitz, & Swann, 2001), such that individuals with poor inhibitory control are more likely to display impulsive behaviour (Dalley, Everitt, & Robbins, 2011; Logan et al., 1997; Schippers et al., 2017; Winstanley, Eagle, & Robbins, 2006). Within species, some individuals behave more impulsively than...
others (e.g. Langley et al., 2020; MacLean et al., 2014; Shaw, 2017), which could have negative consequences for them. More impulsive humans are more likely to develop addictions, have poorer health and lower success in life (e.g. Crews & Boettiger, 2009; Evenden, 1999; Nautiyal, Okuda, Hen, & Blanco, 2017). In other animals, more impulsive individuals are more likely to display stereotypes (e.g. de Haas & van der Eijk, 2018; Garner & Mason, 2002). In addition, higher inhibitory control, and thus lower impulsivity, can improve fitness (in terms of increased reproductive success; Ashton, Ridley, Edwards, & Thornton, 2018; Minter, Keagy, & Tinghitella, 2017). That said, more impulsive behaviour could also be beneficial, for example, if it promotes exploration and, therefore, access to resources. Overall, understanding underlying contributions to observed variation is needed to improve our understanding of the evolution of impulsivity and inhibitory control. Nevertheless, why individuals differ in impulsivity has received little investigation (but see e.g. Langley et al., 2020).

The current lack of explanations for within-species variation in impulsivity may be because impulsivity appears to consist of multiple different aspects in both humans (Evenden, 1999) and other animals (Brucks et al., 2017; Nautiyal, Wall, et al., 2017). These aspects include impulsive action (in which more impulsive individuals are more likely to show some level of impulsivity in at least one task). Therefore, we separated our experimental effects of environmental enrichment, making the effect of cognitive enrichment on impulsivity, and disentangle these from other factors which differed in rearing conditions. These treatments were intended to manipulate aspects of impulsivity, and/or (4) differences in expression of monoaminergic genes. For instance, with repeated experience in a situation, individuals could reduce how impulsively they behave in that situation (i.e. improve their inhibitory control; e.g. Kabadyai, Bobrowicz, & Osvat, 2018; van Horik et al., 2018). The extent to which impulsivity is repeatable over time and to which individuals can adjust their impulsivity with repeated experience will likely affect how much an individual’s impulsivity impacts their survival and fitness. Thus, determining repeatability in, and flexibility of, impulsivity is ecologically and evolutionarily relevant. Whether cognitive or environmental enrichment and variation in genes of the dopaminergic and serotonergic systems influence reduction in impulsivity over repeated experience is unknown. Reduction in impulsivity over repeated experiences could be due to learning (i.e. individuals could learn, from experiencing a situation, to reduce how impulsively they respond to that situation). If so, cognitive enrichment may result in faster reduction in impulsive behaviour, compared to individuals that did not receive cognitive enrichment. That cognitive enrichment has been found to improve cognitive performance (Milgram, 2003; Thornton & Lukas, 2012) supports this hypothesis. Moreover, higher levels of expression of genes from the monoaminergic systems can be associated with better learning performance (Wise, 2004; Strac et al., 2016). Therefore, if a decrease in impulsivity with repeated experience is due to learning, higher serotoninergic and dopaminergic gene expression may cause faster reduction in impulsivity compared to lower serotoninergic and dopaminergic gene expression. Thus, cognitive enrichment and monoaminergic systems may link both to how impulsively individuals initially respond to a relatively novel situation, and how they change their impulsivity over multiple experiences of that situation.

We investigated, in red junglefowl, Gallus gallus, chicks, (1) whether individuals differ in impulsivity, (2) how repeatable such differences are, and whether these differences can be explained by either (3) differences in enrichment received during early rearing and/or (4) differences in expression of monoaminergic genes. For the latter, we focused specifically on the genes DRD1, DRD2, SHT1B, SHT2A, SHT2B, SHT2C and TPH as these genes have previously been linked to impulsivity or inhibitory control. We included two common measures of impulsivity in our study: impulsive action and persistence. We separated our experimental effects of environmental and cognitive enrichment by raising chicks in three treatments which differed in rearing conditions. These treatments were
(1) ‘cognitively enriched’, where chicks participated in cognitive tests and thus gained both cognitive and environmental enrichment, (2) ‘environmentally enriched’, where chicks spent a similar amount of time in testing arenas and interacting with testing equipment as cognitively enriched chicks, without participating in cognitive tests and thus received environmental but not cognitive enrichment, and (3) ‘nonenriched’, where chicks did not enter cognitive testing arenas or interact with testing equipment, and thus received neither cognitive nor environmental enrichment. We tested for differences in gene expression between the three treatments, before exploring links between gene expression and impulsive action, and gene expression and persistence, across treatments. The red junglefowl is a good study animal for cognitive research as they can be raised in relatively large groups, adapt quickly to human handling and are highly motivated to work for food rewards (see Garnham & Løvlie, 2018). Based on previous literature in other species, we expected to see individual variation in, and repeatability of, impulsivity and that individuals would reduce their impulsivity over repeated experience of impulsivity testing. We predicted that cognitive enrichment, specifically, would decrease impulsive action and persistence, and increase the rate of reduction in impulsivity. Regarding monoaminergic genes, we predicted that lower expression levels of dopaminergic and serotonergic genes would be linked to higher levels of both impulsive action and persistence and slower reduction of them.

METHODS

Animals and Housing

We used 133 (N_males = 67, N_females = 56, N_unknown sex = 10) red junglefowl from a pedigree bred population at Linköping University, Sweden. Previous generations of this population have been used in studies of behaviour and cognition (e.g. Garnham, Ahlgren Porthén, Child, Forslind, & Løvlie, 2019; Sorato, Zidar, Garnham, Wilson, & Løvlie, 2018; Zidar, Balogh et al., 2017; Zidar, Sorato et al., 2017; Zidar et al., 2018). Chicks were hatched in artificial incubators, and wing-tagged individually. For the duration of the study, chicks were housed in mixed-sex groups (≤25 individuals) in cages (72 x 71 cm and 53 cm high) equipped with perches, heaters, light (0700–1900 hours) and with ad libitum commercial poultry feed and water. After 9 weeks of age, chicks were moved to a chicken facility outside Linköping (for more information, see Zidar et al., 2017). Chicks were sexed at 6 weeks of age, when they showed no signs of isolation stress (sensu Zidar et al., 2017). To habituate to being around testing equipment reduces stress during impulsivity testing. In this case, as reduced stress can lead to lower impulsivity (Porcelli & Seligman, 2005), we would expect chicks from the cognitively enriched treatment to differ from both the environmentally enriched and nonenriched chicks. Specifically, as cognitive enrichment can improve cognitive performance (Milgram, 2003; Thornton & Lukas, 2012) and impulsivity can be negatively linked to cognitive performance (e.g. Duckworth & Seligman, 2005; Shamosh & Gray, 2008; Vernouillet et al., 2016), we predicted that if cognitive aspects of our cognitive enrichment affected impulsivity, cognitively enriched chicks would be less impulsive than both the environmentally enriched and nonenriched chicks. On the other hand, if noncognitive aspects of cognitive enrichment (such as regular exposure to handling, test arenas and equipment associated with cognitive testing) affected impulsivity more than (or with similar effects to) the cognitive aspects of this enrichment, we would expect chicks from the cognitively enriched and environmentally enriched treatments to differ in a similar way from the nonenriched chicks, but not to be significantly different from each other. This could be the case if habituation to being around testing equipment reduces stress during impulsivity testing. In this case, as reduced stress can lead to lower impulsivity (Porcelli & Delgado, 2008), we would expect both cognitively enriched and environmentally enriched chicks to be less impulsive than nonenriched chicks. Alternatively, as environmental enrichment (with no specific cognitive enrichment) may increase impulsivity (Dalley et al., 2002; Kirkpatrick et al., 2013), both cognitively enriched chicks and environmentally enriched chicks could be expected to be more impulsive than nonenriched chicks, as both these treatments received environmental

Rearing Treatments

To investigate whether differences in enrichment received during rearing influenced impulsivity, we divided chicks randomly into our three rearing treatments and later assayed impulsivity in all chicks using a detour task (described below). We ensured that full-sibling chicks from the same family were spread as evenly as possible between treatments (one to three chicks per family per treatment, N_families = 40). Observers were blind to which treatments chicks were from during testing. The first treatment was our ‘cognitively enriched’ treatment (N = 65). In this treatment, chicks were exposed to a series of reward-based cognitive tests (discriminative and reversal learning tests, cognitive judgement bias test, described below). We considered these tests to be cognitively enriching experiences as they include aspects of training and learning and are known to affect later behaviour in red junglefowl (Zidar et al., 2017) and other species (e.g. Milgram, 2003). The second treatment was our ‘environmentally enriched’ treatment (N = 34). In this treatment, chicks were handled and exposed to the same test arenas, testing equipment and rewards as the cognitively enriched chicks at similar times and for similar durations. These chicks were able to interact with all test equipment used in cognitive tests. However, while chicks from the environmentally enriched treatment gained some enrichment from exploring and interacting with test arenas and equipment, they were never given the specific cognitive enrichment associated with learning that chicks in our cognitively enriched treatment were given (sensu Zidar et al., 2017). Rewards provided to the chicks in the environmentally enriched treatment were scattered randomly around the arena to avoid these chicks learning to associate particular locations in the arena, or test apparatus, with rewards. The third treatment was our ‘nonenriched’ treatment (N = 34); chicks in this treatment were kept in the same housing conditions as the other two treatments but were never handled beyond what was necessary for their care, nor given any experience of equipment used in the cognitive testing. Our chicks remained in these treatments from hatching to when their brains were dissected for gene expression analyses when they were 9 weeks old (see below). The reason for having these three treatments was an attempt to determine whether, if cognitive enrichment affected impulsivity, this was due to either cognitive or noncognitive aspects of our enrichment. If cognitive aspects of our cognitive enrichment (i.e. cognitive stimulation from participating in reward-based learning tasks) specifically affected impulsivity, we would expect chicks from the cognitively enriched treatment to differ from both the environmentally enriched and nonenriched chicks. Specifically, as cognitive enrichment can improve cognitive performance (Milgram, 2003; Thornton & Lukas, 2012) and impulsivity can be negatively linked to cognitive performance (e.g. Duckworth & Seligman, 2005; Shamosh & Gray, 2008; Vernouillet et al., 2016), we predicted that if cognitive aspects of our cognitive enrichment affected impulsivity, cognitively enriched chicks would be less impulsive than both the environmentally enriched and nonenriched chicks. On the other hand, if noncognitive aspects of cognitive enrichment (such as regular exposure to handling, test arenas and equipment associated with cognitive testing) affected impulsivity more than (or with similar effects to) the cognitive aspects of this enrichment, we would expect chicks from the cognitively enriched and environmentally enriched treatments to differ in a similar way from the nonenriched chicks, but not to be significantly different from each other. This could be the case if habituation to being around testing equipment reduces stress during impulsivity testing. In this case, as reduced stress can lead to lower impulsivity (Porcelli & Delgado, 2008), we would expect both cognitively enriched and environmentally enriched chicks to be less impulsive than nonenriched chicks. Alternatively, as environmental enrichment (with no specific cognitive enrichment) may increase impulsivity (Dalley et al., 2002; Kirkpatrick et al., 2013), both cognitively enriched chicks and environmentally enriched chicks could be expected to be more impulsive than nonenriched chicks, as both these treatments received environmental
enrichment (regular experience of arenas and testing equipment), whereas nonenriched chicks did not.

**Cognitive Enrichment**

All cognitively enriched chicks, and only chicks from this treatment, singly took part in discriminative learning tests at 3–4 days old, reversal learning tests at 5–6 days old and cognitive judgement bias tests at 12–13 days old (Fig. A1). All chicks, except one, that took part in these tests passed them. The chick that did not pass was excluded from further testing. Because the aim of these tests was simply to provide cognitive enrichment, the results of these tests are not a focus of this study and will not be discussed.

**Discriminative learning**

In our discriminative learning task (sensu Sorato et al., 2018; Zidar et al., 2017) each chick had to learn to associate a black stimulus (a bowl; 5 cm in diameter and 3 cm high, with a 9 cm² card behind it) with a reward (a piece of mealworm) and a white stimulus (same-sized bowl and card) with no reward. We presented these stimuli simultaneously, and chicks passed this test when they chose, by approaching within 2 cm of it, the rewarded stimulus in six consecutive presentations. To pass this test, we gave each chick up to seven sessions (a session ended when 30 presentations had been made, or ca. 15 min had elapsed, whichever came first) with ≥ 1 h rest between.

**Reversal learning**

In our reversal learning task (sensu Sorato et al., 2018; Zidar et al., 2017) we presented each chick in the cognitive enrichment treatment with the same stimuli as in the discriminative learning task, but now the white stimulus was rewarded and the black not. Each chick was given 3 × 5 min to stop inspecting the black stimulus and approach the white stimulus during their first presentation. Subsequent presentations did not include this opportunity to approach the white bowl, and instead used the same selection criteria as discriminative learning for approaching a bowl. Number of presentations in a session, number of sessions and criteria for passing this task were the same as for discriminative learning.

**Cognitive judgement bias**

In our cognitive judgement bias test (sensu Zidar et al., 2018) chicks were presented with one colour stimulus (a bowl and a card of matching colours) at a time. Colour stimuli were the original white and black, plus an additional three grey, same-sized, cues, that were ambiguous in signal and intermediate in colour to the previously familiar cues (‘light grey’: 75% white/25% black; ‘mid grey’: 50% white/50% black; ‘dark grey’: 25% white/75% black). Chicks were subject to 30 presentations in total with a maximum of 30 s given for each presentation. Only the white stimulus was rewarded, and there was no learning required to pass this test.

**Impulsivity Test**

To measure individual variation in impulsive action and persistence, at 14–19 days of age, chicks from all three treatments were singly exposed to a detour task. The detour task is a well-established test used across species, including birds (Kabadayi et al., 2018; MacLean et al., 2014; Shaw, 2017; Vernouillet et al., 2016), wherein an animal is presented with a reward behind a transparent barrier and must inhibit the impulsive response (reaching straight for the reward) and instead use a learned detour (e.g. Kabadayi et al., 2018; MacLean et al., 2014). The sample sizes for the impulsivity tests were reduced somewhat throughout testing due to natural mortality, or to some chicks not passing the required pretest stages (described below). To prevent differences in reward motivation, we gave chicks in all treatments a similar daily allocation of mealworms prior to this test.

**Pretest stages**

As a prerequisite for participating in the detour task, chicks needed to pass two pretest training stages. First, because familiarity with transparent materials can affect animals’ performance in the detour task (van Horik et al., 2018), we familiarized chicks with transparent materials by having them walk around a three-walled transparent box (13 × 13 cm and 20 cm high), without pecking it, to the open side to find a mealworm reward. Once a chick could do this three times in a row, we considered it to be familiar with transparent materials. Second, we familiarized chicks with the detour action required in our impulsivity test, by using an opaque cylinder (5 cm in diameter and 8 cm long), which they had to approach from the side to retrieve the reward. As chicks could not see the reward in the opaque cylinder, they did not try to peck at the reward through the cylinder at this stage; thus, this stage could not be used to test impulsivity. At the start of each opaque cylinder presentation, we placed the chick and the cylinder at opposite short ends of the arena, with the chick facing the long (i.e. closed) side of the cylinder. We considered each chick to have learned the detour when it retrieved a reward through the side opening of this cylinder five consecutive times. For both pretest stages separately, chicks were given a maximum of five sessions of 15 min, to pass. That all chicks were exposed to the same training prior to the test and only progressed to the test after reaching the same learning criterion should prevent differences in response learning from affecting the test (e.g. as was seen in van Horik, Beardsworth, Laker, Whiteside, & Madden, 2020).

**Test stage**

Chicks that passed the pretest stages took part in a detour task (N aplicación enriched = 57, N environmentally enriched = 28, N nonenriched = 25; N males = 61, N females = 44, N unknown sex = 5). Here, we presented a chick with a transparent cylinder (5 cm in diameter and 8 cm long), containing a visible mealworm, a total of 30 times. Most chicks were given all presentations in one session; however, some needed more sessions (up to three). The position of the cylinder and the chick at the start of each presentation were the same as in the opaque cylinder training stage. During each presentation, we recorded whether, and how many times, the chick pecked on the cylinder towards the reward. While the detour task is typically used to measure inhibitory control, it can also be used to measure impulsivity as this is negatively correlated with inhibitory control (Dalley et al., 2011; Logan et al., 1997; Schippers et al., 2017; Winstanley et al., 2006). For the latter, impulsivity in this test can be measured as the number of times an individual fails to use the detour and tries to reach the reward by pecking directly on the transparent barrier. We measured impulsive action as the number of presentations in which chicks pecked the cylinder (i.e. failed to inhibit the impulsive response) over the first five presentations, whereas persistence was measured as the total number of pecks (no set maximum value) during the first five presentations. We used a cumulative score for persistence to obtain a measure of persistence over the same time period as our measure of impulsive action. We used the first five presentations to avoid the measures of impulsive action and persistence being affected by learning (e.g. Kabadayi et al., 2018). For both impulsive action and persistence, a higher score indicated a higher level of that trait. We used the remaining 25 presentations to investigate variation in reduction of impulsive behaviour with repeated experience of the detour task. To investigate repeatability of impulsivity, 37 chicks were retested in the detour task 1–2 days after they were first tested in this test. The chicks that were used for this investigation of repeatability in
impulsivity took part in 30 presentations of the detour task; however, only the first five presentations were used to investigate repeatability of our measures of impulsivity (see Results).

**Gene Expression Analyses**

To investigate whether gene expression of dopaminergic and serotonergic systems explained variation in impulsivity, we measured brain gene expression of synthesizers and receptors of the dopaminergic and serotonergic systems. These systems were chosen due to their previously detected relationship with impulsivity (Dalley et al., 2002, Strac et al., 2016). We also looked at corticosteroid receptors, specifically glucocorticoid and mineralocorticoid (abbreviated to GR and MR, respectively) to explore whether long-term stress was affecting our results. We culled 83 chicks at 9 weeks old (Ncognitively enriched = 36, Nenvironmentally enriched = 22), by rapid decapitation, followed immediately by brain dissection. The left telencephalon was snap frozen with liquid nitrogen (≤ 4 min) and stored at −80 °C until RNA extraction. We used the left hemisphere as it is the dominant hemisphere for impulsivity (Vallortigara, 1999). Specifically, we used the caudal region, including the nidopallium, for gene expression analyses as this is implicated in impulsivity (Walker, Mikheenko, Argyle, Robbins, & Roberts, 2006).

RNA was extracted using Ambion TRI Reagent (Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. We measured the concentration of extracted RNA using Nanodrop 1000 (Thermo Fisher, Göteborg, Sweden) for all samples, and measured the quality of RNA using Agilent 2100 Bioanalyzer for a pool of cDNA from all individuals. Each 10 μl reaction volume used 1 μg total RNA as a template. The primers used targeted POL2 and TBP for housekeeper genes (sensu Elfwing, Fallahshahroudi, Lindgren, Jensen, & Altimiras, 2014), dopamine receptors DRD1 and DRD2, and serotonin receptors 5HT2A, 5HT1B, 5HT2B and 5HT2C, and serotonin synthesizer TPH (Table A1). To investigate the effect of treatment on long-term stress level, we also included primers for corticosteroid receptors GR and MR (specifically NR3C1 and NR3C2, respectively) as these genes can be influenced by early life stress (Zimmer & Spencer, 2014). Primer specificity was checked by inspecting the melting curve run on pooled cDNA from all individuals. Each 10 μl reaction volume used for the qPCR contained 1 μl of equal parts forward and reverse primer, 60–80 pg cDNA diluted in 2 μl water, 5 μl SYBR Green I Master (Roche Diagnostics, Basel, Switzerland) and 2 μl water. We performed the qPCR in a Light Cycler 480 (Roche Diagnostics) at 5 min 95 °C for activation, followed by 40 cycles (10 s 95 °C, 10 s 60 °C and 20 s 72 °C). The end of the program ran a melting curve from 72 °C to 95 °C, before cooling to 40 °C.

**Statistical Analyses**

We used R version 3.4.3 (R Development Core Team, 2018) for statistical analyses. Unless stated otherwise, sample sizes were 110.

**Individual variation in impulsivity**

Visual inspection (‘car’, Fox & Weisberg, 2011; ‘MASS’, Venables & Ripley, 2002) was used to investigate individual variation in impulsivity. This inspection showed that impulsive action and persistence data followed non-normal distributions; thus, we used nonparametric statistics for our analyses. Because males and females did not differ in our response variables (Mann–Whitney U tests: P > 0.2), we pooled male and female data for further analyses. Despite strong correlations (Spearman rank correlation test: ‘corr.test’, R Development Core Team 2018) between impulsive action and persistence, both across the first five presentations (t5 = 0.920, N = 110, P < 0.001) and all presentations (t5 = 0.940, N = 110, P < 0.001), we analysed both aspects of impulsivity to better improve our overall understanding of factors underlying variation in impulsivity.

**Calculating repeatability of impulsivity**

We calculated the individual repeatability of impulsive action and persistence for individuals across the two test occasions (initial and repeat, ‘rptR’, Stoffel, Nakagawa, & Schielzeth, 2017) for all treatments combined. This was done with a Poisson distribution, 1000 bootstraps and individual ID as a covariate. We also calculated the repeatability for individuals within treatments, again with a Poisson distribution, 1000 bootstraps and individual ID as a covariate, to see whether experimental condition could explain repeatability.

**Effects of treatment on impulsivity**

To investigate the effect of rearing treatment (cognitively enriched, environmentally enriched and nonenriched) on impulsive action, we used a Kruskal–Wallis test and a post hoc Dunn test (‘dunn.test’; Dinno, 2017) on response to the first five presentations of our detour task. To investigate the effect of treatment on persistence, as this variable was overdispersed, we used a general linear model with a negative binomial regression and treatment as a fixed effect (‘MASS’, Venables & Ripley, 2002) on data from the first five presentations of our detour task.

**Effects of treatment on reduction in impulsivity**

To investigate treatment differences in how impulsive action and persistence changed during the 30 presentations of the detour task, we used a generalized linear mixed model with Poisson distribution for impulsive action (‘lm4’, Bates, Mächler, Bolker, & Walker, 2015) and with a negative binomial distribution for persistence (‘MASS’, Venables & Ripley, 2002). We fitted one model each for impulsive action and persistence, with treatment, cylinder presentation (in increments of five: presentations 1–5, 6–10, 11–15, etc. up to 26–30) and an interaction of treatment and presentation as fixed effects, and chick ID added as a random effect.

**Relationships between gene expression and impulsivity**

To investigate how gene expression was related to impulsivity, we first determined gene expression levels of the genes we were interested in. We excluded one of the plates (plate 1) from this analysis due to a calibration error during PCR (remaining birds: Ncognitively enriched = 36, Nenvironmentally enriched = 23, Nnonenriched = 20). We calculated the crossing point (Cp) values over the two housekeeping genes. All samples were run in duplicate, so, for each individual, first we calculated the average expression based on these two values (CV replicates = 12.7%). Then, for each individual, we calculated expression levels of the genes of interest by the difference in expression between the housekeeper genes and the gene of interest (ΔCp). Higher ΔCp values indicate lower gene expression.

Visual inspection of Q–Q plots showed that the distributions of gene expression levels (ΔCp) were log normal; thus, we used parametric statistics. As gene expression could be influenced by treatment, we ran linear mixed models with expression level as response variable and treatment as a fixed effect (‘lm4’, Bates et al., 2015). We included impulsive action score and persistence as fixed effects since these improved model fit based on Akaike information criterion (AIC) scores.

We calculated Pearson rank correlations between gene expression levels (‘Hmisc’, Harrell, 2018). Most of the genes analysed were
correlated with each other \((r > 0.460, N = 79, P < 0.012)\), except for \(TPH\), which did not correlate significantly with any other genes \((r < 0.140, N = 79, P > 0.247)\), and \(NR3C2\) and \(DRD2\) which did not correlate significantly with each other \((r = 0.130, N = 79, P = 0.262)\). Correlated genes were not used in the same model.

Once gene expression was determined, to investigate whether variation in impulsivity was related to gene expression levels, we separately modelled impulsive action and persistence (both measured during the first five presentations of our detour task) as response variable, against gene expression \((\Delta CP)\) as a fixed effect, using generalized linear mixed models with Poisson distribution. In these models, we included two genes that did not correlate \((TPH\) and another gene, done sequentially for all genes) and treatment as fixed effects. We included impulsive action and persistence as fixed effects in the models where they were not the response variable, which improved AIC scores. The results presented had Bonferroni corrections applied to correct for multiple comparisons.

**Ethical Note**

The study (ethical permit number 50-13) was carried out according to Swedish ethical requirements, approved by Linköping Ethical Committee, and followed university and national guidelines for minimizing reduced welfare of research animals.

The chicks used were all housed in pens with equipment that enabled them to perform natural behaviours (perches and sawdust for dustbathing). We took multiple steps to reduce stress and fear in the chicks during testing. First, chicks were habituated to human presence and handling before testing began. Second, unmotivated chicks were given at least 1 h to rest in their home pen before testing continued. Third, we used only reward-reinforced learning.

The number of animals used in total, and killed for brain gene analysis, was high enough for quality investigation at both behavioural and genetic levels without resulting in redundancy. Performing multiple tests using the same individuals reduced the number of animals needed overall.

Euthanasia followed Swedish regulations for, and was carried out by individuals trained in, rapid decapitation.

**RESULTS**

**Individual Variation in and Repeatability of Impulsivity**

Our red junglefowl chicks showed individual variation in both impulsivity and persistence (Fig. A2).

Neither impulsive action nor persistence was repeatable between initial and repeat tests across treatments (original-scale approximation; impulsive action: \(R = 0.140 \pm 0.100, P = 0.193\); persistence: \(R = 0.000 \pm 0.061, P = 0.500\) or within treatments \((R < 0.2, P > 0.3\) for all).

**Effect of Treatment on Impulsivity**

Levels of impulsive action and persistence (measured as responses during the first five presentations of our detour task) differed between our treatments (Fig. 1). Cognitively enriched chicks showed 26.5% higher impulsive action than environmentally enriched chicks \((z_{83} = 1.820, P = 0.070)\) and 44.9% higher impulsive action than nonenriched controls \((z_{80} = 2.640, P = 0.008)\). Cognitively enriched chicks showed 48.1% higher persistence than environmentally enriched chicks \((P_{83} = 0.490, P = 0.038)\) and 61.9% higher persistence than nonenriched chicks \((P_{90} = 0.490, P = 0.010)\). Environmentally enriched chicks and nonenriched control chicks did not differ significantly in impulsive action \((z_{53} = 0.778, P = 0.437)\) or persistence \((P_{53} = 0.150, P = 0.603)\).

**Effect of Treatment on Gene Expression**

The only gene that differed in expression between treatments was the dopamine receptor gene \(DRD2\), which showed lower expression in chicks in the environmentally enriched treatment than in other treatments (Table 2).

**Figure 1.** The effect of rearing treatment on impulsivity of red junglefowl chicks. (a) Impulsive action (number of presentations of a transparent cylinder with a visible reward, in a detour task, in which the chicks pecked the transparent cylinder, 0–5). (b) Persistence (total number of times, over the initial five presentations, in which chicks pecked a transparent cylinder with a visible reward in a detour task, continuous variable). Chicks were either both cognitively and environmentally enriched (black), environmentally but not cognitively enriched (grey) or neither cognitively nor environmentally enriched (white). Values presented are means with SEs. \(*P < 0.1; \quad *P < 0.05\).
Correlation Between Brain Gene Expression and Impulsivity

Chicks that showed higher levels of impulsive action over the first five presentations of our first detour task tended to have higher expression levels of \( \text{DRD2} \) (Table 3, Fig. A3). Chicks that were more persistent during the first five presentations of our first detour task had lower expression levels of the dopamine receptor genes \( \text{DRD1} \) and the serotonin synthesizer gene \( \text{TPH} \) and tended to have lower expression of \( \text{DRD2} \) (Table 3, Fig. A3). Neither impulsive action nor persistence correlated with expression levels of any other genes measured.

**DISCUSSION**

We investigated whether red junglefowl chicks showed individual differences in impulsive action and persistence, whether these differences were repeatable, and whether impulsivity and its change over time were influenced by rearing, specifically in terms of enrichment received (i.e. whether chicks were cognitively enriched, environmentally enriched or nonenriched) or monoaminergic (specifically serotonergic and dopaminergic) gene expression. In doing so, we aimed to improve our understanding of the causes of individual variation in impulsivity and inhibitory control. We found that our junglefowl chicks displayed interindividual variation in impulsivity and that rearing treatment affected impulsive action and persistence in several ways. First, cognitively enriched chicks had higher levels of impulsive action and persistence than nonenriched chicks, whereas environmentally enriched chicks showed similar levels of persistence. That cognitively enriched chicks appear to be more impulsive contradicts our prediction that cognitive enrichment could reduce impulsivity by improving cognitive performance. The only difference in experimental design between cognitively and environmentally enriched chicks is only a tendency, the robustness of this finding needs to be explored further. Cognitively enriched chicks were more persistent than both environmentally enriched and nonenriched chicks, whereas environmentally enriched chicks showed similar levels of persistence that cognitively enriched chicks appear to be more impulsive contradicts our prediction that cognitive enrichment could reduce impulsivity by improving cognitive performance. The only difference in experimental design between cognitively and environmentally enriched chicks was that the cognitively enriched chicks took part in cognitive testing, which included aspects of learning, while environmentally enriched chicks did not. This suggests that participation in cognitive tests (i.e. the cognitive aspect of cognitive enrichment), specifically, could have increased impulsivity in these chicks. This echoes previous work in this population in which cognitive aspects of cognitive stimulation appeared to also affect other behaviours (Zidar et al., 2017). The possible effect of cognitive enrichment on impulsivity in the current study appeared to be similar to the possible effect of environmental enrichment on impulsivity observed in other studies (Dalley et al., 2002; Kirkpatrick et al., 2013). However, our cognitively and environmentally enriched chicks received equivalent amounts of habituation to novel objects and situations (and, therefore, probably, similar amounts of environmental enrichment), yet did not display similar levels of impulsivity. This could indicate that environmental enrichment used in other studies provided sufficient cognitive stimulation (e.g. aspects of social or spatial cognition, Dalley et al., 2002; Kirkpatrick et al., 2013) to influence impulsivity. Further, this indicates that impulsivity is linked to cognitive processes, besides inhibitory control, and can be increased by cognitively enriching experiences. While we here only measured performance in a detour task, future work should explore other potential differences between rearing treatments that could underlie differences in how individuals behave in cognitive tasks. For example, differences in activity or motivation can affect performance in cognitive tasks. This is supported by the finding that, in a later cohort of red junglefowl chicks, measures of activity and distraction (which was

### Table 1

| Response     | Rearing treatment       | Environmentally enriched | Nonenriched control |
|--------------|-------------------------|--------------------------|---------------------|
| Impulsive action | –0.033 (0.005)**        | –0.016 (0.007)*         | –0.031 (0.009)**    |
| Persistence   | –0.033 (0.005)**        | –0.018 (0.012)           | –0.039 (0.003)**    |

Differences in impulsive action (number of presentations of a transparent cylinder with a visible reward, in a detour task, in which the chicks pecked the transparent cylinder, 0–5) and persistence (total number of times in which chicks pecked a transparent cylinder with a visible reward in a detour task, continuous variable) across presentations (1–30, see Methods for details) for the three rearing treatments: cognitively enriched (environmentally and cognitively enriched), environmentally enriched (but not cognitively enriched) and nonenriched control (neither environmentally nor cognitively enriched). Values presented are effect size (\( \beta \)) and SE (in parentheses) from generalized linear mixed models.

*P < 0.05; **P < 0.01.
Figure 2. The effect of rearing treatment on reduction of impulsivity in red junglefowl chicks. The difference across presentations (1–30, as binned data of five presentations) is shown for (a–c) impulsive action (number of presentations of a transparent cylinder with a visible reward, in a detour task, in which the chicks pecked the transparent cylinder) and (d–f) persistence (total number of times in which chicks pecked a transparent cylinder with a visible reward in a detour task). (a, d) Cognitively enriched (environmentally and cognitively enriched), (b, e) environmentally enriched (environmentally but not cognitively enriched) and (c, f) nonenriched (neither environmentally nor cognitively enriched) chicks. Dots represent raw data points, lines are lines of best fit from the models (specified in the Methods) and shadings are SEs.
strongly correlated with food motivation) were linked to how impulsively chicks behaved in a detour task (Garcia Dominguez, Garnham, Thornton, Shaw, Levlie, 2021). Further, stress can often affect performance in cognitive tasks. However, our results do not suggest that variation in chronic stress levels between rearing treatments explains observed differences in impulsivity. This is based on the finding that gene expression levels of glucocorticoid genes NR3C1 and NR3C2 did not differ between chicks in our different treatments. Overall, our results support previous studies (e.g., Dalley et al., 2002; Kirkpatrick et al., 2013), suggesting that individual differences in impulsivity are a result of differences in previous experiences, specifically in terms of enrichment received prior to testing. Future work should aim to explore which aspects of cognitive enrichment affect variation in impulsivity.

With increased experience of the detour task, environmentally enriched chicks tended to reduce their impulsivity more slowly than cognitively or non-enriched chicks. This could indicate that environmentally enriched chicks are slower at learning to reduce impulsivity; nevertheless, further research is needed to confirm this. Environmentally enriched chicks had lower levels of DRD2 gene expression than cognitively enriched or non-enriched chicks. Therefore, if confirmed, this result would support our prediction that lower dopamine brain gene expression would be linked to a slower rate of learning to reduce impulsive behaviour due to the important role of dopamine in learning ( Wise, 2004). That cognitively enriched chicks were not significantly faster at reducing their impulsivity with increased experience of the detour task went against our predictions. Exploring the generality of these findings, the pathway by which they occur and the effect of genetic variation warrants further work. Overall, it is important to keep in mind that, as individuals can alter their impulsivity over trials and may do so at different rates depending on previous experience (e.g. what kinds of enrichment they have received prior to testing), this can complicate interpretations of impulsivity (Kabadayi et al., 2018; van Horik et al., 2018).

Regarding the relationship between impulsivity and monoaminergic gene expression, both impulsive action and persistence were connected to one aspect of serotoninergic system gene expression (TPH expression), albeit in opposite directions. In addition, persistence also correlated with some aspects of dopaminergic brain gene expression (DRD1 and DRD2, with the latter being a tendency). While analyses of brains collected closer in time to the impulsivity test may have detected more variation in gene expression, our results nevertheless suggest that variation in monoaminergic genes can underlie variation in impulsivity. Only DRD2 expression differed between treatments, being expressed at lower levels in environmentally enriched chicks. This indicates that the degree to which environment may influence gene expression can depend on the gene in question. This can, thus, complicate interpretations of how gene expression differences connect to rearing conditions and to behavioural differences.

Impulsive action and persistence were strongly correlated. This could be because they were taken from the same test and thus were not independent measures (similar to Garner & Mason, 2002). Additionally, a chick that scored zero in impulsive action would also score zero in persistence, although, overall, it was theoretically possible for a chick to have a higher impulsive action measure and low persistence (e.g. by only pecking once in each presentation) or low impulsive action but high persistence (e.g. by pecking many times in one presentation and not in others). Despite the correlation between them, impulsive action and persistence were affected by treatment, and related to gene expression, in different ways. This hints that phenotypic correlations between aspects of impulsivity do not necessarily indicate that these aspects share the same underlying mechanisms. For our red junglefowl, our results suggest that impulsive action may be linked more specifically to the serotoninergic system, whereas persistence appears to be linked to both the serotonergic and dopaminergic systems. Nevertheless, further research, investigating links between impulsive action and persistence with other serotonergic and dopaminergic genes would be needed to test this assumption. Regardless, that these aspects of impulsivity seem to relate in different ways to neurological systems supports the hypothesis that impulsivity is a heterogenic trait (Dalley et al., 2011; Nautiyal, Wall, et al., 2017).

In summary, we showed that multiple factors, including previous experiences in terms of type of enrichment experienced during rearing, and gene expression, may contribute to how impulsive individuals are, as well as to their reduction in impulsivity over time interacting with the detour task. Owing to the negative correlation between impulsivity and inhibitory control, these factors could also be expected to influence inhibitory control. Further, we showed that impulsive action and persistence are phenotypically

| Table 2 | Comparison of red junglefowl brain gene expression between rearing treatments |
|---------|-------------------------------|
| **Gene** | **Comparison** | **Gene expression** | **Impulsive action** | **Persistence** |
| DRD1    | C vs N                  | 0.385 (0.321) | 0.102 (0.038) | **0.503 (0.726)** | 0.028 (0.064) | 0.027 (0.060) | 0.054 (0.224) | -0.004 (0.003) | -0.020 (0.085) | 0.013 (0.187) |
| DRD2    | E vs N                  | -0.319 (0.363) | -0.083 (0.043) | **-0.853 (0.821)** | -0.065 (0.072) | -0.084 (0.067) | -0.001 (0.263) | -0.002 (0.004) | -0.055 (0.096) | 0.171 (0.213) |
| 5HT1B   | C vs E                  | 0.066 (0.336) | 0.018 (0.040) | -0.350 (0.760) | -0.037 (0.067) | -0.057 (0.063) | 0.053 (0.246) | -0.006 (0.004) | -0.020 (0.085) | 0.185 (0.196) |
| 5HT2A   | E vs N                  | -0.319 (0.363) | -0.083 (0.043) | **-0.853 (0.821)** | -0.065 (0.072) | -0.084 (0.067) | -0.001 (0.263) | -0.002 (0.004) | -0.055 (0.096) | 0.171 (0.213) |
| 5HT2B   | C vs N                  | 0.066 (0.336) | 0.018 (0.040) | -0.350 (0.760) | -0.037 (0.067) | -0.057 (0.063) | 0.053 (0.246) | -0.006 (0.004) | -0.020 (0.085) | 0.185 (0.196) |
| 5HT2C   | E vs N                  | -0.319 (0.363) | -0.083 (0.043) | **-0.853 (0.821)** | -0.065 (0.072) | -0.084 (0.067) | -0.001 (0.263) | -0.002 (0.004) | -0.055 (0.096) | 0.171 (0.213) |
| TPH     | C vs N                  | 0.066 (0.336) | 0.018 (0.040) | -0.350 (0.760) | -0.037 (0.067) | -0.057 (0.063) | 0.053 (0.246) | -0.006 (0.004) | -0.020 (0.085) | 0.185 (0.196) |
| NR3C1   | E vs N                  | -0.319 (0.363) | -0.083 (0.043) | **-0.853 (0.821)** | -0.065 (0.072) | -0.084 (0.067) | -0.001 (0.263) | -0.002 (0.004) | -0.055 (0.096) | 0.171 (0.213) |

Each row shows data from pairwise comparisons of the effect of treatment on ACP (high ACP indicates lower gene expression). C = cognitively and environmentally enriched, E = environmentally but not cognitively enriched and N = non-enriched control. Comparisons were done using linear mixed models and values presented are effect size (β) with SE (in parentheses). DRD1 and DRD2 refers to gene expression levels of the dopaminergic receptors, ‘5HT1B’, ‘5HT2A’, ‘5HT2B’ and ‘5HT2C’ serotonergic receptors, ‘TPH’ a serotonin synthesizer and ‘NR3C1’ and ‘NR3C2’ corticosteroid receptors. (*p < 0.1; *p < 0.05; **p < 0.01 (Bonferroni corrected).

| Table 3 | Effects of brain gene expression level on impulsivity of red junglefowl chicks |
|---------|--------------------------------------|
| **Gene** | **Impulsive action** | **Persistence** |
| DRD1    | -0.032 (0.06) | 0.089 (0.028) | (*) |
| DRD2    | -0.126 (0.54) | 0.73 (0.29) | (*) |
| 5HT1B   | -0.003 (0.03) | 0.026 (0.016) | |
| 5HT2A   | 0.395 (0.29) | -0.28 (0.18) | |
| 5HT2B   | -0.201 (0.37) | 0.37 (0.21) | |
| 5HT2C   | -0.035 (0.09) | 0.035 (0.055) | |
| TPH     | -18.890 (8.53) | 14.52 (3.050) | |

Gene expression (presented as ACP, where high ACP indicates lower gene expression), differences in relation to impulsive action (number of presentations of a transparent cylinder with a visible reward, in a detour task, in which the chicks pecked the transparent cylinder, 0–5) and persistence (total number of times, over the initial five presentations, in which chicks pecked a transparent cylinder with a visible reward in a detour task, continuous variable). ‘DRD1’ and ‘DRD2’ refers to gene expression levels of dopaminergic receptors, ‘5HT1B’, ‘5HT2A’, ‘5HT2B’ and ‘5HT2C’ serotonergic receptors and ‘TPH’ a serotonin synthesizer. Values presented are effect size (β) with SE (in parentheses) from generalized linear mixed models. (*) p < 0.1; *p < 0.05; **p < 0.01 (Bonferroni corrected).
linked, although seem not to show the same relationships with underlying brain gene expression. Future work should aim to include various aspects of impulsivity, their interrelatedness and potentially underlying genetic and molecular differences, together with the long-term effects of cognitive enrichment on cognition later in life, to improve our understanding of observed variation.

Data Availability

The data collected and analysed for the presented study are provided as supplementary material.

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Supplementary Material

Supplementary material associated with this article can be found online at https://doi.org/10.1016/j.anbehav.2021.06.007.

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Appendix

Table A1

| Target gene | Accession number | Description | Forward primer sequence (5’ to 3’) | Ta (°C) for forward primer | Reverse primer sequence (5’ to 3’) | Ta (°C) for reverse primer | Product length (bp) |
|-------------|------------------|-------------|------------------------------------|-----------------------------|-----------------------------------|---------------------------|---------------------|
| TBP         | NM_205103.1      | TATA box binding protein | TAGCCCGATGATGCCGTAT          | 58.33                       | GTTCCCTGTGTCCCTGCC                | 58.03                     | 147                 |
| POL2        | NM_001006500.1   | RNA polymerase III subunit A | GAGCCTCTTTGCGCCGTTTT         | 58.98                       | GGGGTGATCTTCTTGCTGC               | 56.98                     | 105                 |
| DRD1        | NM_001144848.1   | Dopamine receptor D1    | CAAGCCATTTCACCCCTGC          | 60.04                       | TGGGCTATCTGGCTGGTTAAA             | 60.18                     | 119                 |
| DRD2        | NM_001123290.1   | Dopamine receptor D2    | CATGGCTCTGGCTGGTATAT         | 59.89                       | AGAGCTCTACGGCTGGTGA               | 58.38                     | 497                 |
| 5HT2B       | NM_001172817.1   | Serotonin receptor 1B   | GAAACGACCAAGCTCTCTACA        | 64.01                       | GCCTTCTACGGCTGCTGTC              | 60.66                     | 138                 |
| 5HT2A       | NM_001318420.1   | Serotonin receptor 2A    | GTCAAGGCTGGTCAATG            | 56.50                       | CAGATGGCAGTAAGCA                 | 55.30                     | 155                 |
| 5HT2B       | NM_001290547.1   | Serotonin receptor 2B    | TCAGCTCTCACTGCACTTG           | 60.25                       | TACCCTGGAAAGCTCCTCT              | 60.55                     | 143                 |
| 5HT2C       | NM_001318421.1   | Serotonin receptor 2C    | ACTTGCTTCTCATGCATTCA         | 60.39                       | TCTTGCTTCTCATGCATTCA             | 60.39                     | 175                 |
| TPH         | NM_204956.1      | Tryptophan hydroxylase 1 | ACTCTTTTCTTTTTTCAGCCTTCTT   | 58.00                       | TACCTTCTACGTCTCCATTTACTTC        | 58.32                     | 85                  |
| NR3C1       | NM_00107826.1    | Glucocorticoid receptor | CTTCATCCCCCCTCA             | 56.05                       | TCAGCATTCTGAGCTCGCGC             | 54.29                     | 203                 |
| NR3C2       | NM_001159345.1   | Mineralocorticoid receptor | CAGGAAGCTCAAGGAATG          | 59.44                       | GTTCTACCATGACCTTGGG             | 58.57                     | 92                  |

Primer information for primers used in this study. Target gene: name of the gene targeted by PCR (POL2 and TBP are housekeeper genes; the others are target genes). Accession number: unique bioinformatics number given to DNA sequence. Description: full gene name of the target genes. Forward primer sequence: DNA sequence of the forward primer, 5’ to 3’. Ta for forward primer: annealing temperature for the forward primer sequence, °C. Reverse primer sequence: DNA sequence of the reverse primer, 5’ to 3’. Ta for reverse primer: annealing temperature for the reverse primer sequence, °C. Product length: the length of the sequence obtained with the primer in base pairs.

Figure A1. Timeline of the behavioural tests the red junglefowl were exposed to, from time of hatching until culling.

Figure A2. Histograms of the frequency of red junglefowl chicks’ responses to a detour task. Red: cognitively and environmentally enriched chicks; green: environmentally enriched chicks; blue: nonenriched control chicks. (a) Impulsive action: number of presentations of a transparent cylinder with a visible reward, in a detour task, in which the chicks pecked the transparent cylinder, 0–5. (b) Persistence: total number of times a chick pecked at a transparent cylinder with a visible reward, in a detour task, over the first five presentations, continuous variable.
Figure A3. Effect of gene expression levels on impulsivity in red junglefowl chicks. Only significant or trending interactions between gene expression and impulsivity are shown. Impulsive action: number of presentations of a transparent cylinder with a visible reward, in a detour task, in which the chicks pecked the transparent cylinder, 0–5. Persistence: total number of times, over the initial five presentations, in which chicks pecked a transparent cylinder with a visible reward in a detour task, continuous variable. (a) TPH gene expression and impulsive action, (b) TPH gene expression and persistence, (c) DRD1 gene expression and persistence, (d) DRD2 gene expression and persistence. Gene expression is shown as ΔCP (where high values indicate lower expression).