Investigating the influence of brood size manipulation on exploratory behavior in great tits *(Parus major)*

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Abstract

Personality is often defined as a set of correlated behavioral traits that are consistent across time and space. In great tits, personality is frequently measured by the degree of exploratory behavior where birds lay between a continuum of slow to fast exploratory behavior. Exploratory behavior can be influenced by an interplay between several factors such as experience (the older the bird, the more experiences it may gain with predation or social), morphology, genetics and early developmental circumstances (e.g. food availability and brood size). However it is only partly known how the behavior is formed. Therefore, the main aim of this study was to investigate the influence of brood size manipulation on exploratory behavior in a wild-caught great tit population (*Parus major)* in the Netherlands. In addition to that, the relationship between body weight and exploratory behavior and the relationship between age and exploratory behavior was analyzed as well. Brood size was manipulated to create reduced, enlarged and unmanipulated brood sizes. A cross-foster experiment was carried out to distinguish between the environmental and genetic influences on the exploratory behavior. The results of this study showed no significant effect of brood size manipulation on exploratory behavior. Moreover, no significant relationship between body weight and exploratory behavior was found. However, a significant positive relationship between age and exploratory behavior was found, which may be caused by morphological changes that come with age or that older birds may have more experience. In conclusion, based on this study, exploratory behavior might not be significantly influenced by brood size manipulation and the single morphological trait body weight. However, a lack of evidence in this study does not mean that exploratory behavior is not influenced by these factors. Therefore, for future studies, it would be interesting to also take sex and the sex ratio within nests regarding exploratory behavior into account. Moreover, it would be recommended to also investigate the survival of the birds from the different treatments (reduced and enlarged) of the brood size manipulation since this may affect the sample size of the study.

Introduction

In the animal kingdom, it has been shown that there are consistent differences between individuals in how they react during novel, social or stressful situations (Verbeek et al. 1994; Sih et al. 2004; Carere et al. 2005a). This can be differences in reactivity, boldness and shyness and can be clustered together with other behavioral traits, such as fearfulness, aggression and risk-taking behavior. These clusters of traits form the personality of an animal (Carere et al. 2005a). The personality of an animal is often defined as a set of correlated behaviors that is

consistent across time and space (Sih et al. 2004; Carere et al. 2014). Personality in birds is frequently measured by the degree of exploratory behavior where a bird may express behavior that lies between a continuum of slow to fast exploratory behavior (Carere et al. 2014). Exploratory behavior can be linked with different traits such as aggression, risk-taking behavior and differences in physiological reactions to stressful situations (Naguib et al. 2011). Fast birds express more proactive behavior while slow individuals are more reactive in response to a novel environment (Verbeek et al. 1994). In great tits (*Parus major*), it has been shown that aggressiveness and exploratory behavior are correlated in individuals in a laboratory setting (Verbeek et al. 1994; Verbeek et al. 1999) and in the wild (Hollander et al. 2008). How the personality of an animal is influenced by different factors is only partly known.

Exploratory behavior can be influenced by an interplay between several factors, such as genetic factors (Ledón-Rettig et al. 2013; Verhoeven et al. 2016), hormones (Stöwe et al. 2010; Martins et al. 2007), changes in the environment (e.g. food availability and climate), experiences during life (Renner 1987) and early developmental circumstances (e.g. brood size, habitat quality, food) (Dingemanse & De Goede 2004; Naguib et al. 2011; van Overveld et al. 2014). Very early parental effects (such as thermoregulation during incubation of the eggs) could also play a role in shaping the phenotype of the offspring (Reed & Clark 2011). Maternal hormones, which are transported from the mother to the eggs and embryos may also play a role in shaping behavior of the offspring (Gil 2008; Reed & Clark 2011). For example, in the study of Martins et al. (2007), the exploratory speed and risk-taking behavior in zebra finches was investigated. They bred selection lines for different levels of corticosterone hormones (high, low and control). The researchers showed that differences in corticosterone levels were correlated with differences in exploratory behavior and risk-taking behavior. A higher level of corticosterone hormone resulted in a higher score for exploratory behavior (Martins et al. 2007). Moreover, parents may also influence the phenotype of their young via the brood size within the nest.

Naguib et al. (2011) conducted an experiment where they manipulated the brood size (reduced or control brood sizes) of great tits and investigated the influence of the social environment (group size) on personality traits and stress response. They used two artificial selection lines that differ in exploratory behavior and they used birds from a wild origin (hand reared) which nested in nest boxes in the Netherlands. Stress response was measured by a handling stress-test in which they measured breath rate before and after social isolation. They found a strong stress response in individuals that were raised in a small brood. The stress response of birds from a wild population was not influenced by brood size. However, the researchers did not find a significant relationship between exploratory behavior and brood size manipulation. The researchers took also sex and sex ratio within the nests into account in their analysis and investigated the sex-specific differences in exploratory behavior. They found that birds raised in a female-biased brood had a higher exploratory score than birds from a male-biased brood (Naguib et al. 2011).

Another factor that may influence personality is the age of an animal. This may be due to morphological changes that come with age or that older individuals may have had more experience with different situations (Stamps & Groothuis 2010). For example, a study in rats showed that exploratory behavior may be influenced by previous experiences. Rats that were placed in an environment which allows for interaction with objects change their behavior when novel objects are investigated (Renner 1987). However, a study in hand-reared great tits (selection lines for fast and slow) showed that individuals who were tested for exploratory behavior at the age of 4-6 weeks, had similar scores at the age of 6 months. Therefore, the researchers suggest that social experience may not influence the exploratory score in hand-reared great tits (Drent & Marchetti 1999).

Moreover, morphology (such as body weight, growth rate, body size and bill length) of birds may also play a role in explaining variation in exploratory behavior (Verbeek et al 1996; Carere et al. 2005b; Moiron et al 2019). These morphological traits may be influenced by early circumstances, such as brood size and the provisioning of food which is regulated by the parents of the offspring. In turn, the rate of provisioning by the parents is affected by offspring demand and the availability of food resources (Low et al. 2012). The brood size within a nest may also influence the distribution of food by parents to each offspring. Parents may not be able to fully compensate for a larger brood and therefore nestling quality might be lower when compared to smaller brood sizes (Hõrak et al. 2003). Moreover, in larger brood sizes, the intensity of begging by nestlings is increased due to an increase in sibling competition, which may affect the body condition of the birds (Neuenschwander et al. 2003). In the study of Carere et al. (2005b), the influence of food availability and sibling competition on personality in great tits was investigated. The researchers used two different lines that were selected for fast and slow exploratory behavior. Via a food rationing protocol, they created an impairment in the growth rate, thereby decreasing the body weight of the birds, and induced an increase of begging of the offspring. Overall, food-rationed birds (both from the fast and slow line) had a high exploratory score in the novel environment and novel object test when compared to their parents or the non-food rationed animals (control). This was especially pronounced in the slow line when compared with their parents. Not only was there a change in exploratory score, also an increase in aggression in birds originating from the fast line was found (Carere et al. 2005b).

Another study related to morphological traits and behavior was performed by Moiron et al. (2019). The researchers conducted a study to investigate the relations between body weight and risk-taking behavior in great tits. The researchers studied covariation patterns between morphology and behavior. For this, they used body weight, bill, tarsus and wing length as morphology traits and aggressivity and exploratory behavior as behavioral traits (which form together the behavioral syndrome “risk-taking behavior”). Subsequently, the role of these 6 traits were investigated and whether these traits were linked with one certain phenotype. The researchers found two latent variables (body-size dependent behavior and size-independent variation in body weight (also known as body condition)) that could be integrated when explaining behavior and morphology. They found that birds that took more risk, were larger individuals. On the other hand, they found that individuals that explored their territory more had a lower body condition (body weight that is independent of body size) when compared to individuals that avoided risk. The researchers hypothesize that this could be due to secure more resources. Another explanation might be that birds that weigh more may be slower due to a higher wing-load and thus may be easier to predate upon (Moiron et al. 2019).

Ultimately, these behavioral studies may give more insight in how the personality of animal is formed and eventually may influence the survival and reproductive success. Differences in personality may be linked to differences in foraging strategies or the capability to dominate other individuals while competing for food, mates or territory (Drent & Marchetti 1999; Dingemanse, & De Goede 2004). There also might be a trade-off involved when looking at differences in coping strategies. Some individuals may be more active when a predator is present. This may result in more time spend foraging or searching for mates, however there will be an increased risk of getting predated (Sih et al. 2004; Nácarová et al. 2018). Thus, the personality of an animal is crucial for its survival and reproductive success. However, it is only partly known how the personality is formed. In the study of Naguib et al. (2011) they also investigated the effect of brood size manipulation on exploratory behavior. However, in this study they used hand-reared birds. The researchers hand-reared birds in the laboratory from day

10 on. Moreover, they only reduced the brood size and compared it with a control brood and did not investigate the effects of an enlargement of brood size on behavior. In this study, birds will be raised in a natural setting. A study in with European starlings showed that hand-reared birds might be less fearful and impulsive (Feenders & Bateson 2013). Thus, hand-rearing in birds might induce behavioral changes which can be avoided when nestlings are raised by their genetic or foster parents. Moreover, the brood size will be enlarged as well to compare both reduced and enlarged brood sizes with a control brood.

The main focus of this study is on the early development environment (brood size) in relation to exploratory behavior in wild great tits (see appendix 1 for introduction on DNA methylation and epigenetics). Therefore, the main goal of this study is to investigate the influence of brood size manipulation on exploratory behavior in a wild great tit population in the Netherlands. It is hypothesized that birds raised in large broods will be faster in exploratory behavior than birds raised in small broods. Individuals that were raised in a high-density population may express more bold and aggressive behavior due to competition between birds for food or territory (Naguib et al. 2011). On the other hand, birds that are raised in a small group may become less aggressive and less fast due to less competition for food or mates (Neuenschwander et al. 2003; Naguib et al. 2011). Thus, great tits raised in large broods may be faster in exploratory behavior than birds raised in small broods.

In addition to investigating the influence of brood size manipulation on exploratory behavior, the relationship between body weight and exploratory behavior in great tits will be analyzed. Firstly, it is expected that the enlargement of the brood size will result in nestlings with a lower body weight. This may be due to an increase of competition between siblings, which may be more in larger brood sizes. Consequently, the energy costs for the nestlings due to more begging is higher which may affect the body condition of the nestlings (Neuenschwander et al. 2003). Moreover, the parents may be not able to fully compensate for the enlargement of the brood size which may also lead to a decrease in body weight (Nur N. 1984; Hõrak 2003). In addition to that, it is expected that individuals with a lower body weight, and thus individuals from a larger brood size, will be faster in exploratory behavior than heavier animals based on outcome based on the studies of Moiron et al. (2019) and Carere et al. (2005b). Lastly, the relationship between age and exploratory behavior will also be investigated. It is expected that there might be a positive relationship between the age of an individual and exploratory behavior because older individuals may have had more time to gain more experiences or due to morphological changes that come with age (Carere et al. 2005b; Stamps & Groothuis 2010).

Methods

Study species and area

The great tit (*Parus major*) is a small nonmigratory passerine bird that is common all over the world. It breeds in natural cavities and artificial nest boxes throughout Europe and parts of Asia. The bird is mostly monogamous and territorial during the breeding season (Kvist et al. 2003). For this study, a wild great tit population from the Netherlands at the Westerheide area near Arnhem (5°500E, 52°000N) in 2018 was used. Westerheide is an area, around 250 ha that consists of a mixed wood, such as birch and oak trees (Dingemanse & de Goede 2004). In this area, great tits nest in nest boxes (n=250) which are attached to tree trunks. The breeding season in the Netherlands ranges from April to June, where most chicks hatch in the beginning/mid of May (Carere et al. 2005). Nest boxes were checked once a week to monitor the breeding and hatching stages of the birds. Two days before hatching, nest boxes were checked daily.

Cross-foster experiment

A cross-foster experiment was done to distinguish between the environmental effect and genetic influences on the behavior (van Oers et al. 2015). Day 0 was the day of hatching. On day 1 or 2, birds were weighed and marked by down coding to recognize the individuals. Two broods with the same hatch date (pair of broods), similar weight and brood size were paired with each other. To decide whether a brood pair became a control or a treatment (reduced or enlarged brood size) pair a coin was flipped (heads = enlarged, tails = reduced). The chicks within a nest box were ranked on weight (high to low) and given a rank number (heaviest bird was given rank one, second heaviest rank 2 etc.). This was done to avoid the chance that, for example, all the heavy individuals went to the same nest box. Subsequently, the chicks with odd numbers went to one nest box and the chicks with even numbers went to the other nest box. This was determined by tossing a coin (heads= all odd number to nest box x, tails=all even chicks to nest box x). The same was done for the pairs that received the treatment. However, this pair of broods consisted of a reduced and an enlarged brood. This was also determined by tossing a coin. Birds within nest boxes were ranked again on weight and given a rank number. Birds with odd numbers went to one nest box and birds with even numbers went to the other. To create reduced (- 3 chicks) or enlarged (+3 chicks) nests excel was used. Chicks (that were ranked on weight) were randomly chosen by excel under the condition that the ratio remains 50:50 between foster and genetic siblings within one brood. To remove or add 3 chicks was chosen because the resulting number will still lay within the range of natural variation of clutch sizes (Boyce & Perrins 1987). In addition to that, parents are not able to fully compensate for the enlargement of the brood size (Hõrak 2003). The total study set-up consisted out of 64 nest boxes (control: n chicks =236 (32 broods), reduced: n chicks=83 (16 broods) and enlarged: n chicks =140 (16 broods)) and in total 461 individuals. On day 6, birds were weighed and ringed and on day 14, birds were weighed again.

Mist netting

Capturing started only after two weeks after the last chicks had fledged (end of June till second week of September), to assure the chicks were nutritional independent in case either the chicks themselves or the parents were caught. Birds were captured with mist nets at feeding sites and brought to the Netherlands Institute of Ecology (NIOO-KNAW) at Wageningen. The mist nets

were set-up three times a week. After capture, birds were individually held in cages of 0.9 x 0.4 x 0.5 m. The cage consisted of a solid bottom, top and side with a wire-mesh in front. Birds were provided with ad libitum water and food (mealworms, egg food, sunflower seeds). The body weight of the birds was also measured. The next morning, a novel environment test was done (which will be explained below). After the test, birds were released back at Westerheide.

Novel environment test

The novel environment test that was used was based on the method described in Dingemanse et al. (2002). Birds were individually held in a cage that was connected with the novel environment test room. They were tested between 0800 and 1500 hours. When the test started, a towel was placed over the cage and the door was opened that connected the cages and novel environment test room. The novel environment test room was lit with artificial light and contained 5 artificial wooden trees and an observer window. When the door of the cage opened, the bird had 2 minutes to get out of the cage and the time was noted down. If the bird did not leave the cage after 2 minutes, it was chased out by lifting the towel. The test lasted for a maximum of 10 minutes and exploratory behavior was measured by noting the different behaviors (pecking, landing on a tree, hopping, landing on a wall, and landing on the window, door or ground) down. After maximum 10 minutes, the lights were turned off and the bird was returned to its cage.

Statistical analysis

For the statistical analysis, the program RStudio 1.3.959 (R. Development Core Team 2008) for Windows was used. The packages ‘ggplot’, ‘lme4’, ‘multcomp’ and ‘lmerTest’ were used to analyse the exploratory data of 2018. The birds used for the analysis were the birds that were captured during mist netting and where the exploratory score was measured from: control: n birds =50, enlarged: n birds =28 and reduced: n birds=42 (27 brood pairs which consisted of 12 control pairs and 15 treatment pairs (reduced or enlarged). Exploratory data of 2018 was checked for normality. Subsequently, Linear mixed models (LMM) were constructed to investigate the influence of brood size manipulation on exploratory behavior. Exploratory score consisted out of the sum of n times hopping and n times landing on tree or wall corrected for season (see methods Novel environment test; see the paper of Dingemanse et al. 2002 for further explanation on correction for season). Two models were constructed. Firstly, a model to test for the random structure fitted with Restricted Maximum Likelihood (REML). This was to investigate the effect of cross-fostering. This model consisted out of exploratory score as dependent variable and random factors nest box of rearing and nest box of origin (nested in cross-foster pair). Secondly, a model to test for the fixed factors by using Maximum Likelihood (ML) was constructed. This model consisted of exploratory score as dependent variable, treatment (control, reduced and enlarged) as fixed factor and both nest box of rearing and origin (nested in cross-foster pair) as random factors (see table 1).
Subsequently, an analysis of variance (anova) was performed to test which of the two models fit the data the best and to confirm the results that were found in the LMM’s. For this, ML was used for both models, to test for the fixed factors. Lastly, a Dunnett’s post-hoc was performed to compare the means of the treatments (reduced and enlarged) with the control mean.

Moreover, the influence of the treatment on body weight on day 14 was investigated with a LMM. Again, two models were constructed. The first model consisted of body weight

on day 14 as dependent variable and nest box of origin and nest box of rearing (nested in cross-foster pair), and pair as random factors. Pair was added separately to correct for an effect of date on the body weight of the birds. A second model was constructed with body weight on day 14 as dependent variable, treatment (control, reduced and enlarged) as fixed factor and nest box of rearing, nest box of origin (nested in pair) and pair as random factors (see table 1). An anova was carried out to investigate which model fitted the data the best. Subsequently, a model was constructed to investigate the relationship between the body weight (before testing) and the exploratory score. A model was constructed with exploratory score as dependent variable and nest box of origin and nest box of rearing (nested in pair) as random factors. The second model consisted of exploratory score as dependent variable, weight before testing as fixed factor and nest box of origin and nest box of rearing (nested in pair) as random factors (see table 1). Subsequently, an anova was carried out to compare these two models with each other to investigate which model fits the data best.

Lastly, the relationship between age (testing date – hatching date) and exploratory score was investigated. Two models were constructed again. The first model consisted of exploratory score as dependent variable and nest box of rearing and nest box of origin as random factors (nested in pair). The second model contained exploratory score as dependent variable, age as fixed factor and nest box of rearing and nest box of origin (nested in pair) as random factors. An anova was conducted to compare both models (see table 1). For all the models used in this study, a significance level of 0.05 was used.

Table 1: overview of the different LMM's used in this study. NK = nestkast (nestbox)

exploratory behavior and brood size manipulation	
model 1	Exploratory score ~ 1+1|Pair: RearingNK + 1|Pair: OriginNK, REML = TRUE
model 2	Exploratory score ~ Treatment+ 1|Pair: RearingNK + 1|Pair: OriginNK, REML = FALSE
body weight day 14 and brood size manipulation	
model 3	Weight_14 ~ 1 + 1|Pair: RearingNK + 1|Pair: OriginNK + 1|Pair, REML = TRUE
model 4	Weight_14 ~ Treatment + 1|Pair: RearingNK + 1|Pair: OriginNK + 1|Pair, REML = FALSE
exploratory behavior and body weight (before testing)	
model 5	Exploratory score ~ 1 + 1|Pair: RearingNK + 1|Pair: OriginNK, REML = TRUE
model 6	Exploratory score ~ Weight before testing + 1|Pair: RearingNK + 1|Pair: OriginNK, REML = FALSE
exploratory behavior and age	
model 7	Exploratory score ~ 1 + 1|Pair: RearingNK + 1|Pair: OriginNK, REML = TRUE
model 8	Exploratory score ~ age + 1|Pair: RearingNK + 1|Pair: OriginNK, REML = FALSE

Results

Effect of brood size manipulation on exploratory behavior

Exploratory data of 2018 was checked for normality and followed (after z-transformation) a normal distribution (see appendix 2). A summary of the data of 2018 can be found in table 2 and a visualisation of the means of the different treatments with standard errors in figure 1.

Table 2: Summary statistics of exploratory data 2018: mean, standard deviation (sd) and standard error (se) for the three different treatments: control, enlarged or reduced brood size.

treatment	n	mean	sd	se
control	50	16.5	6.06	0.86
enlarged	28	17.43	7.1	1.34
reduced	42	17.58	4.07	0.63

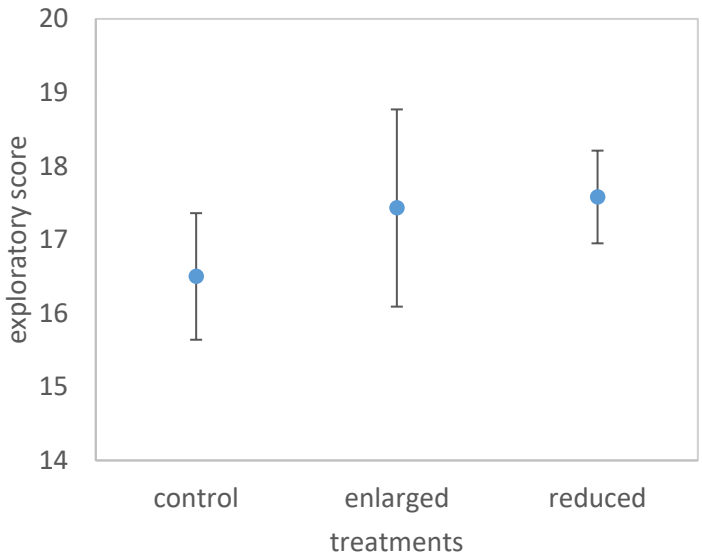

Figure 1: Exploratory score in birds from control, reduced and enlarged brood sizes. Values are means with standard errors.

Subsequently, LMMs were used to analyse the effect of brood size manipulation on exploratory behavior in great tits. The first model showed that there was no variance explained by the nest box of origin (table 3; note that the random factor 'nest box of origin' may include genetic factors and very early circumstances such as maternal hormones or thermoregulation during incubating. Moreover, it is not 100 % certain that the chicks raised in nest box of origin were raised by their biological parents). However, nest box of rearing explains 2.2 % of the variance and 97.8% of the variance was residual variance.

Table 3: LMM model 1 results. n = 120 (genetic n = 56, foster n = 46). Signif. codes: 0 '***'

fixed effect	estimate	std. error	df	t value	p-value	variance	std. dev.
intercept	17.10	0.53	38.29	32.16	$<2e^{-16***}$	-	-
random effect							
origin	-	-	-	-	-	0.00	0.00
rearing	-	-	-	-	-	0.71	0.84
residual	-	-	-	-	-	31.55	5.62

The second model showed that both treatments enlarged (p-value = 0.50) and reduced (p-value=0.38) had no significant effect (table 4) on exploratory behavior (see appendix 2 for full models and models with random effects without nested in pair).
The two models were compared with an anova to investigate which model has the best fit. The model with treatment and the model without treatment did not significantly differ from each other (Anova, df = 2, x^2 (2) = 0.88, p = 0.64). Therefore, the anova also confirmed that there is no significant effect of brood size manipulation on the exploratory score.

Table 4: LMM model 2 results of testing the influence of treatment on exploratory score (dependent variable) n = 120 (genetic n = 56, foster n = 46). Signif. code: 0 '***'

fixed effect	**estimate**	**std. error**	**df**	**t value**	**p-value**	**variance**	**std. dev.**
enlarged	0.92	1.34	36,45	0.69	0.50	-	-
reduced	1.06	1.19	35.72	0.89	0.38	-	-
intercept	16.51	0.81	36.05	20.51	$<2e^{-16***}$		
random effect							
origin	-	-	-	-	-	0.0	0.0
rearing	-	-	-	-	-	0.17	0.42
residual	-	-	-	-	-	31.81	5.64

Lastly, a Dunnett's post hoc was carried out to compare the means of the treatments (reduced and enlarged) with the mean of the control. Both the enlarged (p=0.72) and the reduced (p=0.85) group did not significantly differ from the control group.

Effect of brood size manipulation on body weight

Subsequently, the effect of brood size manipulation on body weight was investigated. In figure 2, the mean body weight (g) of the birds on day 14 with standard errors is visualized per treatment.

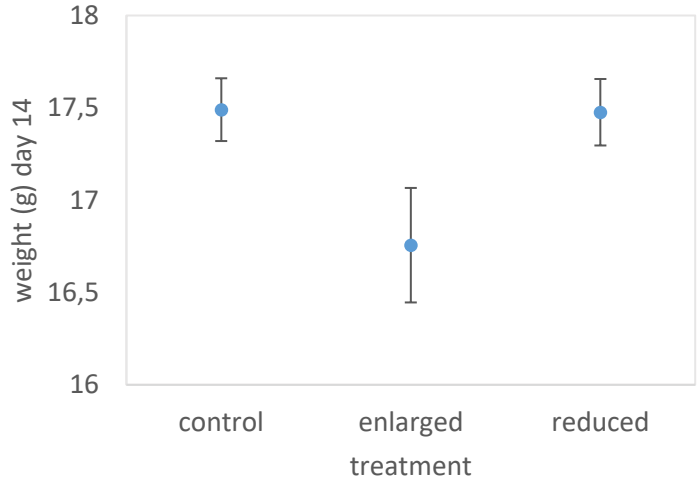

Figure 2: Body weight (g) of day 14 in great tits (reduced: n = 36, enlarged: n = 23, control: n = 43). Values are means with standard errors.

A LMM was carried out to investigate the influence of treatment (reduced and enlarged) on body weight on day 14. Again, in the model to test for random effects, no effect of environment of origin (0% variance) was found (table 5). However, 43,86% of the variance was explained by an effect of rearing environment, 15.2% was explained by cross-foster pair and 40.93% was residual variance.

Table 5: LMM model 3 results. n = 105 (origin n = 50, rearing n = 41). Signif. codes: 0.00 '***'

fixed effect	estimate	std. error	df	t value	p-value	variance	std. dev.
intercept	17.15	0.188	31.54	91.24	$<2e^{-16***}$	-	-
random effect							
origin	-	-	-	-	-	0.00	0.00
rearing	-	-	-	-	-	0.75	0.87
pair	-	-	-	-	-	0.26	0.51
residual	-	-	-	-	-	0.70	0.84

No effect of treatment on weight on day 14 was found (LMM, reduced: p = 0.64, enlarged: p = 0.099; table 6). The comparison between the two models also showed no significant differences (Anova, df 1, x^2 (1) =0.20, p = 0.11; for full LMM results see appendix 3).

Table 6: LMM model 4 results of influence of treatment on weight day 14 (dependent variable). n = 105 (origin n = 50, rearing n = 41). Signif. codes: 0.00'***'

fixed effect	estimate	std. error	df	t value	p-value	variance	std. dev.
enlarged	-0.77	0.46	36.04	-1.69	0.099	-	-
reduced	0.18	0.39	36.79	0.47	0.64	-	-
intercept	17.26	0.28	30.75	61.86	$<2e^{-16***}$		
random effect							
origin	-	-	-	-	-	0.0	0.0
rearing	-	-	-	-	-	0.56	0.75
pair	-	-	-	-	-	0.30	0.55
residual	-	-	-	-	-	0.70	0.83

Relationship between body weight and exploratory behavior

Two linear mixed models were constructed. The first model (to test for the random structure) showed again that there was no variance explained by the environment of origin. Rearing environment explains 2.36% and 97.64% of the variance is residual variance (table 7).

Table 7: LMM results model 5. n = 105. 1. Signif. codes: 0 '***

fixed effect	estimate	std. error	df	t value	p-value	variance	std. dev.
intercept	17.04	0.56	34.11	30.6	$<2e^{-16***}$	-	-
random effect							
origin	-	-	-	-	-	0.00	0.00
rearing	-	-	-	-	-	0.73	0.85
residual	-	-	-	-	-	30.14	5.49

No significant correlation was observed between the weight before testing (fixed factor) and the exploratory score (dependent variable) either (LMM, p = 0.063; table 8; for full LMM results see appendix 4).

Table 8: LMM results model 6 testing the weight before exploratory test and exploratory score. n = 105.

fixed effect	**estimate**	**std. error**	**df**	**t value**	**p-value**	**variance**	**std. dev.**
weight before testing	0.93	0.49	102.97	1.88	0.064	-	-
intercept	1.40	8.36	102.86	0.17	0.87	-	-
random effect							
origin	-	-	-	-	-	0.36	0.60
rearing	-	-	-	-	-	0.60	0.78
residual	-	-	-	-	-	28.64	5.35

The model with weight before testing as fixed factor and the model without the weight did not significantly differ from each other (Anova, df = 1, x^2 (1) = 3.39, p = 0.066; appendix 3). See figure 3 for visualisation of the data.

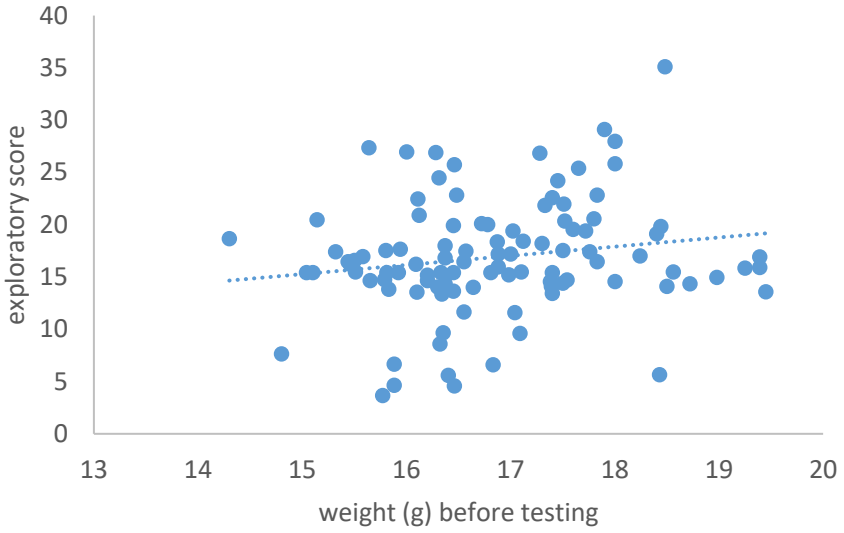

Figure 3: Graph of exploratory score against measured body weight (g) before performing the novel environment test (n=105).

Relationship between age and exploratory behavior

Lastly, the relationship between age and exploratory behavior of the great tits was analysed. The first model (to test for the random structure) showed that 0% is explained by nest box of origin. 2.36% of the variance is explained by nest box of rearing and 97.64% of the variance is residual variance (table 9; for results of models with age (without correction for date) see appendix 5).

Table 9: LMM results model 7. n = 120 (genetic n = 56, foster n = 46). Signif. codes: 0 '***'

fixed effect	**estimate**	**std. error**	**df**	**t value**	**p-value**	**variance**	**std. dev.**
intercept	17.04	0.56	34.11	30.6	$<2e^{-16***}$	-	-
random effect							
origin	-	-	-	-	-	0.00	0.00
rearing	-	-	-	-	-	0.73	0.85
residual	-	-	-	-	-	30.15	5.49

The second model where age was incorporated showed a significant relationship between age and exploratory score (LMM, p = 0.0013; table 10; for full LMM results see appendix 5).

Table 10: LMM results model 8. n = 120 (genetic n = 56, foster n = 46). Signif. code: 0 '***' 0.001'**'

fixed effect	**estimate**	**std. error**	**df**	**t value**	**p-value**	**variance**	**std. dev.**
age	0.055	0.017	104.90	3.30	0.0013**	-	-
intercept	12.57	1.44	96.19	8.70	$8.53e^{-4***}$		
random effect							
origin	-	-	-	-	-	0.00	0.00
rearing	-	-	-	-	-	0.043	0.21
residual	-	-	-	-	-	27.65	5.26

A comparison between both models showed also a significant difference (Anova, df = 1, x^2 (1) = 10.35, p = 0.0013). In figure 4, a graph was made to visualize the relationship between age and exploratory behavior (see appendix 5 for additional information about the interaction between treatment and age).

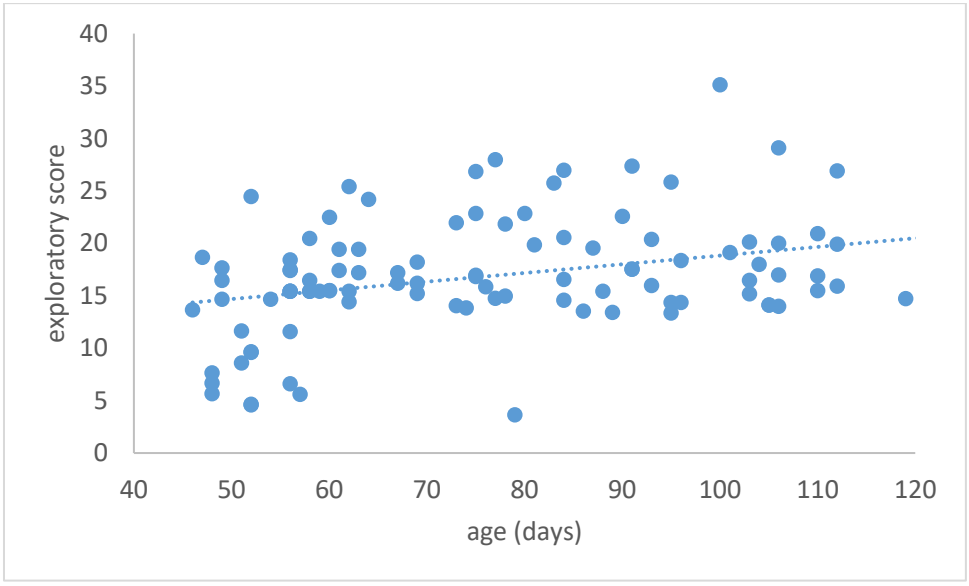

Figure 4: relationship between exploratory score and age of the birds (testing date – hatching date) (n=120).

Discussion

The main aim of this study was to investigate the influence of brood size manipulation on exploratory behavior in a wild great tit population in the Netherlands. In addition to that, the relationship between body weight and exploratory behavior as well as the relationship between exploratory behavior and age was investigated. It was shown that brood size manipulation did not significantly influenced the exploratory behavior of the birds. No significant relationship between body weight and exploratory behavior was found. However, the relationship between age of the birds and exploratory behavior was significant. It was found that older birds had a higher exploratory score. Moreover, the random structure of the models was also investigated. It was found that in all three models the variance explained by nest box of origin was 0%. This means that the variance in exploratory score is for a large part independent of the nest box of origin. Thus, the cross-fostering experiment is effective to distinguish between the environmental and genetic (and very early circumstances) influences on the behavior. On the other hand, it is expected that a percentage of variance would be explained by the nest box of origin since exploratory behavior is heritable (Drent et al. 2003). However, a large part of the variance was assigned to residual variance. This might be due to the fact that within a cross-foster pair there need to be enough chicks from both the nest box of origin and the nest box of rearing. If this is not the case, the model cannot accurately estimate the distribution of variances across the different random factors and therefore may assign most of the variance to residual variance.

Brood size manipulation and exploratory behavior

This study did not reveal a significant effect of brood size manipulation on exploratory behavior. This was in contrast with the initial hypothesis that birds from a larger brood size would be faster in exploratory behavior than birds originated from smaller brood sizes. These results are in line with the findings of the study of Naguib et al. (2011), where they also did not find a significant relationship between brood size manipulation (reduced and control) and exploratory behavior. The researchers found an increase in response to stress in great tits from selection lines and smaller brood sizes. This suggests that brood size may play a role in stress responses to certain environmental circumstances. Moreover, they discovered that sex ratio might have an influence on exploratory behavior. Individuals that were raised in a female-biased nest, were faster in exploring than individuals raised in male-biased broods. This might indicate that the composition rather than the size of the brood may influence the exploratory behavior of great tits (Naguib et al. 2011).

When conducting the cross-foster experiment and brood-size manipulation, the chicks were assigned to different nests without knowing the sex of the birds. As a consequence, the sex ratio within nest boxes might change. As described above, chicks raised in a female-biased brood appear to be more exploratory than chicks raised in a male-biased brood (Naguib et al. 2011). In this study, the sex of most birds was unknown, therefore it is not known how the cross-fostering changed the sex ratios in this experimental set-up.

The small sample size of the different treatments could also have influenced the results. Small sample sizes may increase variability and therefore may lead to biases. Moreover, the birds that received the treatment reduced or enlarged had a higher mean exploratory score when compared to the control. However, the sample size of the different treatments was not equal

(control: n = 50, enlarged: n = 28 and reduced: n = 42). Both for the reduced and enlarged group had a smaller sample size than the control which may also lead to a bias. It is known that different circumstances during fledging may affect the survival chance of the birds later in life Thus, changing the brood size of the birds, and therefore altering the optimal brood size, may affect parental investment and optimal weight of fledging and therefore the survival of the birds (Tinbergen & Boerlijst 1990; Burness et al. 2000). Moreover, in enlarged brood sizes the competition (increase of begging activity) between chicks is increased. Therefore, the energetic costs of begging will be higher in enlarged brood sizes compared to smaller brood sizes and this may lead to a reduced body condition (Neuenschwander et al. 2003).

Body weight and exploratory behavior

No significant relationship between body weight and exploratory behavior was found. This was in contrast with the initial hypothesis which was that individuals with a lower body weight be faster in exploratory behavior than heavier individuals.

Firstly, the brood size manipulation did not significantly affect the body weight on day 14 of the birds. However, only the birds that were weighed on day 14 and that were tested for the exploratory score were included in the analysis. Therefore, a significant effect may have been found if all the birds in this study were included.

Moreover, Moiron et al. (2019), investigated the relationship between body condition and exploratory behavior and found that more exploratory individuals had a lower body condition than individuals that were avoiding risks. Moreover, they found that individuals that were taking more risk, were larger than risk-avoiding individuals. This suggest that not the body weight itself may influence the behavior of the birds but a combination of variables such as differences in tarsus, wing-length and bills together could influence exploratory behavior (Moiron et al. 2019).

Age and exploratory behavior

A significant relationship between the age of the birds and exploratory behavior was found. It was found that the exploratory score became higher with older age.
Carere et al. (2005a) conducted a study with selection lines for fast and slow individuals and investigated the stability and consistency of behavior in great tits over a 2-3-year time spawn. Here, they found that when looking at the overall selection lines, behavior was consistent over time. However, when they looked at individuals, behavior was less consistent across time and space. It was observed that the birds from the slow selection lines became faster in adulthood for the novel object test (Carere et al. 2005a). This was also observed in in the study of Verbeek et al. (1994), where they also investigated the consistent individual differences in hand-reared male great tits. They observed that birds that were tested in a novel object test became faster when tested again 9 weeks later. Moreover, in rats it was also shown that previous experience may change the exploratory behavior (Renner 1987). However, as stated before, it should be kept in mind that the sample size was not equal for the different treatments and therefore the results may be biased.

In conclusion, exploratory behavior in great tits was not significantly influenced by brood size manipulation in this study. Moreover, no significant relationship between body weight and

exploratory behavior was found. However, the relationship between age and exploratory behavior was found to be significant. A lack of evidence for the influence of brood size and body weight in this study does not mean that exploratory behavior is not influenced by these factors. Therefore, in the future, it would be interesting to also take the sex of the birds into account as well as identifying the sex ratio within nests when constructing models. Moreover, when investigating morphological traits consider taking other morphological variables into account as well such as bill and wing length and tarsus. Lastly, it would be recommended to investigate the differences in survival rate between the different treatments (control, enlarged and reduced) as this may affect the sample size and bias the results and to take this into account when creating models. The findings from this study indicate that it is a challenge to disentangle the different factors that may influence the personality, since it is more likely that an interplay between several factors may eventually shape the personality of an animal.

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References

Berthouly, A., Helfenstein, F., & Richner, H. (2007). Cellular immune response, stress resistance and competitiveness in nestling great tits in relation to maternally transmitted carotenoids. *Functional Ecology*, *21*(2), 335–343. https://doi.org/10.1111/j.1365-2435.2006.01236.x

Boyce M.S., & Perrins M. (1987). Optimizing Great Tit Clutch Size in a Fluctuating Environment. *Ecology*, (Vol. 68, Issue 1, pp. 142-153), http://www.jstor.com/stable/1938814

Burness, G. P., McClelland, G. B., Wardrop, S. L., & Hochachka, P. W. (2000). Effect of brood size manipulation on offspring physiology: An experiment with passerine birds. *Journal of Experimental Biology*, *203*(22), 3513–3520.

Carere, C., Drent, P. J., Privitera, L., Koolhaas, J. M., & Groothuis, T. G. G. (2005a). Personalities in great tits, Parus major: Stability and consistency. *Animal Behavior*, *70*(4), 795–805. https://doi.org/10.1016/j.anbehav.2005.01.003

Carere, C., Drent, P.J., Koolhaas J.M. (2005b). Unravelling animal personalities: How and why individuals consistently differ. In *Behavior* (Vol. 142, Issue 9, pp. 1149–1157). https://doi.org/10.1163/156853905774539436

Carere, C., Maestripieri, D., Oers, K. Van, & Marc, N. (2014). Avian Personality. *Animal Personalities*, (April 2015), 66–95. https://doi.org/10.7208/chicago/9780226922065.003.0004

Class, B., & Brommer, J. (2020). Contrasting multilevel relationships between behavior and body mass in blue tit nestlings. *Behavioral Ecology*, 1–9. https://doi.org/10.1093/beheco/araa014

Dall, S. R. X. (2004). Behavioral biology: Fortune favours bold and shy personalities. *Current Biology*, *14*(12), 470–472. https://doi.org/10.1016/j.cub.2004.06.011

Dingemanse, N. J., Both, C., Drent, P. J., Van Oers, K., & Van Noordwijk, A. J. (2002). Repeatability and heritability of exploratory behavior in great tits from the wild. *Animal Behavior*, *64*(6), 929–938. https://doi.org/10.1006/anbe.2002.2006

Dingemanse, N. J., & De Goede, P. (2004). The relation between dominance and exploratory behavior is context-dependent in wild great tits. *Behavioral Ecology*, *15*(6), 1023–1030. https://doi.org/10.1093/beheco/arh115

Drent, P. J., & Marchetti, C. M. (1999). Individuality, exploiration and foraging in hand raised juvenile Great Tits. In N. J. Adams, & R. H. Slotow (Eds.), *Proceedings 22 Int. Ornithol. Congr., Durban* (pp. 896-914). BirdLife South Africa

Drent, P. J., Van Oers, K., & Van Noordwijk, A. J. (2003). Realized heritability of personalities in the great tit (Parus major). *Proceedings of the Royal Society B: Biological Sciences*, *270*(1510), 45–51. https://doi.org/10.1098/rspb.2002.2168

Feenders, G., & Bateson, M. (2013). Hand rearing affects emotional responses but not basic cognitive performance in European starlings. *Animal Behavior*, *86*(1), 127–138. https://doi.org/10.1016/j.anbehav.2013.05.002

Fucikova, E., Drent, P. J., Smits, N., & Van Oers, K. (2009). Handling stress as a measurement of personality in great tit nestlings (parus major). *Ethology*, *115*(4), 366–374. https://doi.org/10.1111/j.1439-0310.2009.01618.x

Gil, D. (2008). Chapter 7 Hormones in Avian Eggs: Physiology, Ecology and Behavior. *Advances in the Study of Behavior*, *38*(08), 337–398. https://doi.org/10.1016/S0065-3454(08)00007-7

Herborn, K. A., Heidinger, B. J., Alexander, L., & Arnold, K. E. (2014). Personality predicts behavioral flexibility in a fluctuating, natural environment. *Behavioral Ecology*, *25*(6), 1374–1379. https://doi.org/10.1093/beheco/aru131

Hollander, F. A., Van Overveld, T., Tokka, I., & Matthysen, E. (2008). Personality and nest defence in the great tit (Parus major). *Ethology*, *114*(4), 405–412. https://doi.org/10.1111/j.1439-0310.2008.01488.x

Hõrak, P. (2003). When to pay the cost of reproduction? A brood size manipulation experiment in great tits (Parus major). *Behavioral Ecology and Sociobiology*, *54*(2), 105–112. https://doi.org/10.1007/s00265-003-0608-1

Ledón-Rettig, C. C., Richards, C. L., & Martin, L. B. (2013). Epigenetics for behavioral ecologists. In *Behavioral Ecology* (Vol. 24, Issue 2, pp. 311–324). https://doi.org/10.1093/beheco/ars145

Lindström, J. (1999) Early development and fitness in birds and mammals. Trends in Ecology&Evolution, 14, 343–348.

Low, M., Makan, T., & Castro, I. (2012). Food availability and offspring demand influence sex-specific patterns and repeatability of parental provisioning. *Behavioral Ecology*, *23*(1), 25–34. https://doi.org/10.1093/beheco/arr145

Martins, T. L. F., Roberts, M. L., Giblin, I., Huxham, R., & Evans, M. R. (2007). Speed of exploration and risk-taking behavior are linked to corticosterone titres in zebra finches. *Hormones and Behavior*, *52*(4), 445–453. https://doi.org/10.1016/j.yhbeh.2007.06.007

Moiron, M., Araya-Ajoy, Y. G., Mathot, K. J., Mouchet, A., & Dingemanse, N. J. (2019). Functional relations between body mass and risk-taking behavior in wild great tits. *Behavioral Ecology*, *30*(3), 617–623. https://doi.org/10.1093/beheco/ary199

Nácarová, J., Veselý, P., & Fuchs, R. (2018). Effect of the exploratory behaviour on a bird's ability to categorize a predator. *Behavioural Processes*, *151*(October 2017), 89–95. https://doi.org/10.1016/j.beproc.2018.03.021

Naguib, M., Flörcke, C., and Van Oers, K. (2011). Effects of social conditions during early development on stress response and personality traits in great tits (Parus major). *Developmental Psychobiology*, *53*(6), 592–600. https://doi.org/10.1002/dev.20533

Neuenschwander, S., Brinkhof, M. W. G., Kölliker, M., & Richner, H. (2003). Brood size, sibling competition, and the cost of begging in great tits (Parus major). *Behavioral Ecology*, *14*(4), 457–462. https://doi.org/10.1093/beheco/arg025

Nur. N (1984). The Consequences of Brood Size for Breeding Blue Tits II . Nestling Weight , Offspring Survival and Optimal Brood Size, *Journal of Animal Ecology , Vol . 53 , No . 2 (Jun ., 1984), pp . 497-517 Published by : British Ecol*. *53*(2), 497–517.

van Oers, K., Kohn, G. M., Hinde, C. A., & Naguib, M. (2015). Parental food provisioning is related to nestling stress response in wild great tit nestlings: Implications for the development of personality. In *Frontiers in Zoology* (Vol. 12, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/1742-9994-12-S1-S10

van Overveld, T., Careau, V., Adriaensen, F., & Matthysen, E. (2014). Seasonal- and sex-specific correlations between dispersal and exploratory behavior in the great tit. *Oecologia*, *174*(1), 109–120. https://doi.org/10.1007/s00442-013-2762-0

R. Development Core Team (2008) R: A language and environ- ment for statistical computing. Vienna, Austria: R Founda- tion for Statistical Computing.

Reed, W. L., & Clark, M. E. (2011). Beyond maternal effects in birds: Responses of the embryo to the environment. *Integrative and Comparative Biology*, *51*(1), 73–80. https://doi.org/10.1093/icb/icr032

Renner, M. J. (1987). Experience-dependent changes in exploratory behavior in the adult rat (Rattus norvegicus): Overall activity level and interactions with objects. *Journal of Comparative Psychology*, *101*(1), 94–100. https://doi.org/10.1037/0735-7036.101.1.94

Sih, A., Bell, A., & Johnson, J. C. (2004). Behavioral syndromes: An ecological and evolutionary overview. *Trends in Ecology and Evolution*, Vol. 19, pp. 372–378. https://doi.org/10.1016/j.tree.2004.04.009

Stamps, J., & Groothuis, T. G. G. (2010). The development of animal personality: Relevance, concepts and perspectives. *Biological Reviews*, *85*(2), 301–325. https://doi.org/10.1111/j.1469-185X.2009.00103.x

Stöwe, M., Rosivall, B., Drent, P. J., & Möstl, E. (2010). Selection for fast and slow exploration affects baseline and stress-induced corticosterone excretion in Great tit nestlings, Parus major. *Hormones and Behavior*, *58*(5), 864–871. https://doi.org/10.1016/j.yhbeh.2010.08.011

Tinbergen, J.M., & Boerlijst, M . C . (1990). Nestling Weight and Survival in Individual Great Tits (Parus major), Journal of Animal Ecology , Vol . 59 , No . 3, pp . 1113-1127 Published by : British Ecological Society St. 59(3), 1113–1127.

Verbeek, M. E. M., Drent, P. J., & Wiepkema, P. R. (1994). Consistent individual differences in early exploratory behavior of male great tits. *Animal Behavior*, *48*(5), 1113–1121. https://doi.org/10.1006/anbe.1994.1344

Verbeek, M. E. ., Boon, A., & Drent, P. J. (1996). Exploration , Aggressive Behavior and Dominance in Pair-Wise Confrontations of Juvenile Male Great Tits Author (s): Monica E . M . Verbeek , Anne Boon and Piet J . Drent Published by : BRILL Stable URL : http://www.jstor.org/stable/4535403 . EXPLORATIO. *Behavior*, *133*(11), 945–963.

Verbeek M.E.M., de Goede, P., Drent, P.J & Wiepkema P.R. (1999). Individual behavioral characteristics and dominance in aviary groups of great tits. *Behavior*, *136*(1), 23-48.

Verhoeven, K. J. F., VonHoldt, B. M., & Sork, V. L. (2016). Epigenetics in ecology and evolution: What we know and what we need to know. *Molecular Ecology*, *25*(8), 1631–1638. https://doi.org/10.1111/mec.13617

Appendix 1 introduction epigenetics, DNA methylation and epiGBS (also methods)

Due to the circumstances with the Corona crisis, I was not able to continue with the project about epigenetics (DNA methylation) in relation to exploratory behavior and brood size manipulation which was initially my focus. Therefore, my aim of this project is now only focused on the influence of brood size manipulation in relation to exploratory behavior. In addition to that, I investigated the relation between body weight of the birds and exploratory behavior as well as the relationship between age and exploratory behavior.

In appendix 1 I put my initial introduction about epigenetics and DNA methylation and methods of epiGBS that was already finished.

Variation in exploratory behavior can be the result of genetic effects and especially the influence of particular genes (van Oers et al. 2004; Rodenburg and van Oers 2010). In the paper of Drent et al. (2003), an experiment was described to investigate the relationship between genetics and personality traits in great tits. They used a four generations of bi-directional artificial selection lines and a wild bird population. They tested the animals collected from the field for exploration of a novel environment whereas the hand-reared young were tested first for novel environment exploration and then for novel objects. The researchers focused on the heritability of early exploratory behavior and found that there was a heritable component in a wild bird population.

Candidate genes for variation of exploratory behavior in vertebrates are dopamine receptor D4 (DRD4) and serotonin transporter (SERT) (Fidler et al. 2007; Riyahi et al. 2015; Verhulst et al. 2016). In humans, the DRD4 receptor is associated with differences in individuals in novelty-seeking behavior (Munafò et al. 2008) and it has implications in human pathologies such as attention deficit hyperactivity disorder (Qian et al. 2018). Another study showed that a single nucleotide polymorphism (SNP) in exon 3, SNP830, of the DRD4 gene may be related to differences in exploratory behavior in wild caught birds raised in the laboratory from one population of great tits (Korsten et al. 2010). The SERT gene has also been proposed to have implications in variations in behavior (Killu et al. 2018; Riyahi et al. 2015). In humans, the gene might play a role in anxiety and other behavioral disorders (Gross and René 2004). A study with great tits showed that the SERT gene is involved in novelty seeking behavior. Here, they found that a polymorphism in the SERT gene, SNP234, was significantly associated with novelty seeking behavior in captive birds. However, the researchers could not conclude that there was a significant relation between the DRD4 gene and personality (Riyahi et al. 2015).

Phenotypic plasticity, which is defined as the capacity of an organism to change its phenotype according to the environmental circumstances (Relyea 2005). However, the underlying mechanism behind this is unclear. Epigenetic mechanisms may play a role in explaining differences in exploratory behavior. Epigenetics can be described as the study of epigenetic marks in the genome. These marks are produced by biochemical mechanisms that can change gene expression by influencing transcription or translation without creating alterations in the genome. These epigenetic changes involve histone modification, microRNAs and DNA methylation. Epigenetic processes in the genome can be induced by environmental circumstances (Bossdorf et al. 2008; Jaenisch and Bird 2003; Verhoeven et al. 2016). It is hypothesized that epigenetics play a role in phenotypic plasticity (Verhoeven 2016). Moreover, epigenetic mechanisms may underlie variation in phenotype that cannot be linked to a difference in DNA sequences between individuals (Bossdorf 2008; Sepers 2019).

The most studied epigenetic mark is DNA methylation (Bossdorf 2008). This is a mechanism where there is an attachment of a methyl group, mainly, to a cytosine (Sepers 2019). This modification mainly takes place at CG dinucleotides, which are present as short DNA sequences called CpG islands (CGI). The methylation of cytosine may inhibit initiation of DNA transcription in the promotor and thereby change the expression of certain genes (Auclair and Weber 2012). In great tits, it has been shown that there are differences in the degree of methylation in genes that are tissue-specific from brain and blood samples. Researchers observed less methylation at transcription start sites (TSS). Moreover, they found differences in methylation of CGIs in promotor regions that are related to development (Derks et al. 2016). DNA methylation in relation to exploratory behavior may play an important role in certain genes, such as the DRD4 gene, as mentioned earlier (Verhulst et al. 2016). In the paper of Verhulst et al. (2016), they describe a possible indication that DNA methylation in the DRD4 gene promotor is associated with variation in animal personality and especially exploratory behavior in great tits. The researcher found a difference in DNA methylation levels in the DRD4 gene in artificially selected breeding lines of great tits that vary in exploratory behavior.

Additionally, there is evidence that epigenetic alterations can be transgenerational (Richards 2006) and this may influence the evolutionary capacity of phenotypic traits in species (Sepers 2019). In the study of Zimmer et al. (2017), the researchers investigated these transgenerational effects in quails. They show that exposing the mother to pre-natal stress has an influence on traits on their offspring. Even more, the offspring inherited the same stress-coping traits at all phenotypic characteristics (neuroendocrine, physiological and behavior traits) that they investigated (Zimmer et al. 2017).

Brood size differences may also play a significant role in variation in overall levels of DNA methylation. Sheldon et al. (2018) found that natal brood size was positively correlated with the percentage of DNA methylation in blood. Moreover, they found that in DNA of birds that were raised in a manipulated brood size occurred more demethylation at early development compared to birds raised in broods that remained the same size. The cause of this variation may be explained by sibling competition and this can result in differences in fitness, physiology and behavior, such as exploratory behavior, in adulthood (Sheldon et al. 2018).

Ultimately, there is increasing evidence that factors such as environment and brood size may influence DNA methylation and thereby may alter exploratory behavior in animals. A better understanding of epigenetics will give us a better insight in the ability of organisms to adapt to the currently changing environment and climate. Moreover, it may help elucidate the underlying mechanism of phenotypic variation among populations (Bossdorf 2008). Therefore, the aim of this study is to investigate DNA methylation levels in the DRD4 and SERT gene in relation to exploratory behavior within great tit families. This will be done via brood-size manipulation experiments. Furthermore, we will investigate and compare DNA methylation levels between great tits with differences in body weights. I expect that DNA methylation levels will be highest for both the DRD4 and SERT gene in individuals that express more exploratory behavior and that are raised in larger broods (Carere et al. 2005; Riyahi et al 2015; Verhulst et al. 2016).

To analyze the genome for DNA methylation levels, we used epigenotyping by sequencing (epiGBS) (van Gurp et al. 2016). This technique is a promising alternative for representation bisulfite sequencing. EpiGBS targets mostly CG rich regions, for example promotor regions, of the DNA via restriction enzymes and size selection. The samples can be pooled together after the digestion and adapter ligation, which makes it low cost per sample (Gurp et al. 2016; Sepers 2019). Moreover, for this study it is particularly an attractive technique because of the big population size of the great tits. Due to the pooling of samples, the sample size of this study

remains of a considerable size.

Methods EpiGBS

To identify the methylated cytosines in the genome, the method epiGBS based on the paper of van Gurp et al. (2016) was used (for full protocol see appendix 1). We used 360 samples and divided them over 10 libraries. Samples were chosen in such a way that there were approximately 4 parents and 12 offspring in one library (3 foreign offspring and 3 own offspring and 2 parents for each nest). Moreover, only complete nests were included. For example, when one parent died or was missing, we excluded the whole nest from the analysis.

DNA was isolated and the concentrations was quantified with the Nanodrop. Subsequently, a DNA digestion was performed with MspI and Nsil overnight. Adapters were annealed and ligated overnight with T4 DNA ligase and nonphosphorylated adapters to minimize the formation of dimers. A quantitative polymerase chain reaction (qPCR) was done to measure the concentration of the samples. Subsequently, samples were pooled and concentrated with the Nucleospin Gel & PCR cleanup kit. DNA concentration was checked again and size selection was performed. Size selection was done with solid-phase reversible immobilization (SPRI) beads. Nick repair was done and a test PCR was performed. Then a bisulphite conversion was done to convert unmethylated cytosines to uracil, whereas cytosines that are methylated remain the same. Subsequently amplification and cleaning of the library was performed. To remove adapter dimer products, a SPRI clean-up was done and the concentration of the library was quantified. The DNA samples were send for sequencing to Novogene Hong Kong.

Appendix 2 Analysis of the effect of brood size manipulation on exploratory behavior

Checking for normality

To investigate if the data was normal distributed, the residuals of the exploratory data 2018 were plotted in a histogram (figure 1). Overall, the data was normal distribute after the data was z-transformed (figure 2).

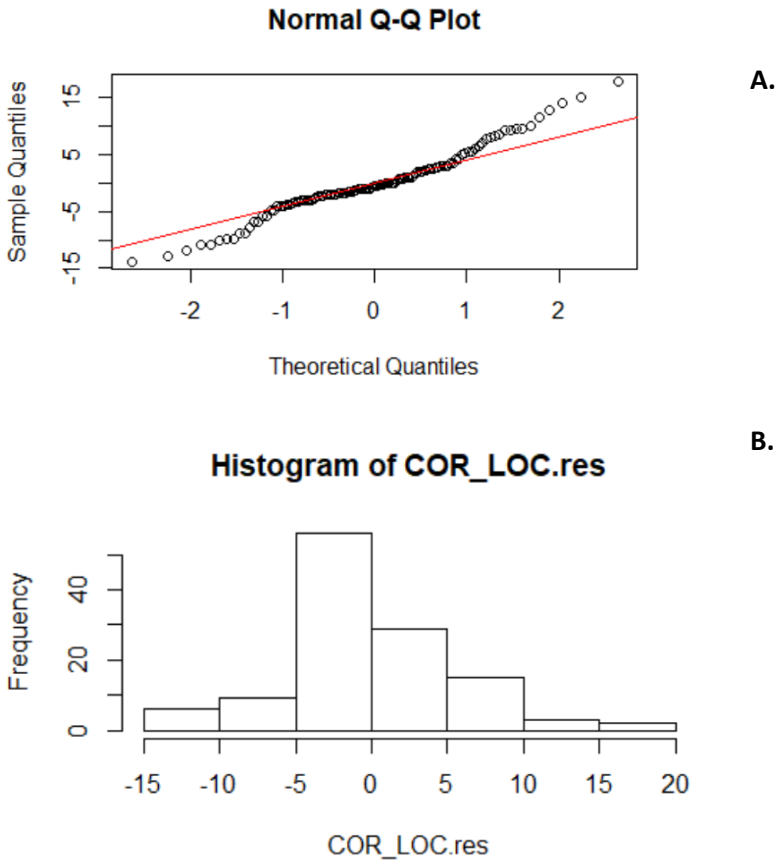

Figure 1 : **a)** qq plot of the residuals (exploratory data 2018) **b)** histogram of residuals of exploratory score (exploratory data 2018).

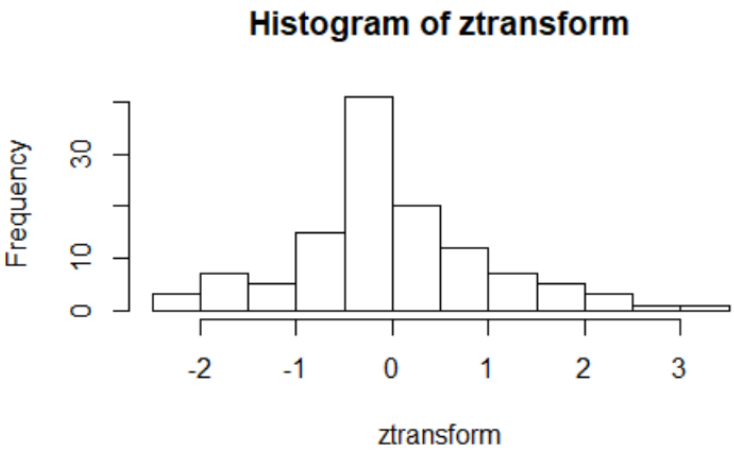

Figure 2: histogram of z-transformed values (exploratory data 2018).

Subsequently, an LMM was constructed to investigate the influence of the brood size manipulation on exploratory score (figure 3) and compared the two models to investigate which model best fitted the data (figure 4) with anova. A Dunnett's post hoc was performed to compare the treatment with the control (figure 5).

Moreover, the same LMM models without the random factors GeneticNK and FosterNK (note that throughout the appendix Genetic NK = nest box of origin and FosterNK = nest box of rearing) nested in pair were constructed, to investigate if there was a difference (figure 6) between the nested and the not nested models. However, no big difference was observed between the models and the anova also yielded almost the same results as the models where the random factors where nested in pair (figure 7). However the random structure slightly changed as a small part of the variance was assigned to nest box of origin.

A.

```
Linear mixed model fit by REML. t-tests use Satterthwaite's method [
lmerModLmerTest]
Formula: COR_LOC ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
   Data: Mydata

REML criterion at convergence: 756.7

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.4004 -0.4463 -0.1131  0.4592  3.2008

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept)  0.0000  0.000
 Pair:FosterNK  (Intercept)  0.9682  0.984
 Residual                   31.5542  5.617
Number of obs: 120, groups:  Pair:GeneticNK, 56; Pair:FosterNK, 46

Fixed effects:
            Estimate Std. Error      df t value Pr(>|t|)
(Intercept)  17.1042     0.5388 37.9089   31.75   <2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
convergence code: 0
```

B.

```
Linear mixed model fit by maximum likelihood . t-tests use
  Satterthwaite's method [lmerModLmerTest]
Formula:
COR_LOC ~ Treatment + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
   Data: Mydata

     AIC      BIC   logLik deviance df.resid
   768.4    785.1   -378.2    756.4      114

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.4435 -0.5013 -0.1055  0.4761  3.1354

Random effects:
 Groups         Name        Variance  Std.Dev.
 Pair:GeneticNK (Intercept) 1.170e-07 0.0003421
 Pair:FosterNK  (Intercept) 1.735e-01 0.4165355
 Residual                   3.181e+01 5.6398154
Number of obs: 120, groups:  Pair:GeneticNK, 56; Pair:FosterNK, 46

Fixed effects:
                  Estimate Std. Error      df t value Pr(>|t|)
(Intercept)        16.5085     0.8050 36.0469  20.507   <2e-16 ***
TreatmentEnlarged   0.9243     1.3436 36.4584   0.688    0.496
TreatmentReduced    1.0651     1.1915 35.7264   0.894    0.377
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 3: models for testing the influence of the brood size manipulation on exploratory score. **A)** linear mixed model for testing random factors FosterNK and Genetic NK nested in pair. **B)** linear mixed model for testing influence of treatment (brood size manipulation) on exploratory behavior.

```
Models:
lmer1: COR_LOC ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
lmerT: COR_LOC ~ Treatment + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
      npar    AIC    BIC  logLik deviance  Chisq Df Pr(>Chisq)
lmer1    4 765.24 776.39 -378.62   757.24
lmerT    6 768.36 785.08 -378.18   756.36 0.8808  2     0.6438
```

Figure 4*:* anova between LMM with treatment and without treatment.

```
Fit: lmer(formula = COR_LOC ~ Treatment + ((1 | Pair:FosterNK) + (1 |
    Pair:GeneticNK)), data = Data2, REML = FALSE)

Linear Hypotheses:
                         Estimate Std. Error z value Pr(>|z|)
Enlarged - Control == 0   0.9243     1.3436   0.688    0.725
Reduced - Control == 0    1.0651     1.1915   0.894    0.585
(Adjusted p values reported -- single-step method)
```

Figure 5: Dunnett's post hoc test between enlarged and reduced compared with the control.

A.

```
Linear mixed model fit by REML. t-tests use Satterthwaite's method [
lmerModLmerTest]
Formula: COR_LOC ~ 1 + ((1 | FosterNK) + (1 | GeneticNK))
   Data: Mydata

REML criterion at convergence: 756.2

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.3586 -0.4763 -0.1330  0.4715  3.1869

Random effects:
 Groups    Name        Variance Std.Dev.
 GeneticNK (Intercept)  1.012   1.006
 FosterNK  (Intercept)  1.107   1.052
 Residual              30.409   5.514
Number of obs: 120, groups:  GeneticNK, 45; FosterNK, 41

Fixed effects:
            Estimate Std. Error      df t value Pr(>|t|)
(Intercept)  17.0628     0.5613 32.5815    30.4   <2e-16 ***
```

B.

```
Formula: COR_LOC ~ Treatment + ((1 | FosterNK) + (1 | GeneticNK))
   Data: Mydata

     AIC      BIC   logLik deviance df.resid
   768.1    784.8   -378.0    756.1      114

Scaled residuals:
     Min       1Q   Median       3Q      Max
-2.42623 -0.48984 -0.07875  0.48521  3.14450

Random effects:
 Groups    Name        Variance Std.Dev.
 GeneticNK (Intercept)  0.7918  0.8898
 FosterNK  (Intercept)  0.6784  0.8237
 Residual              30.4953  5.5223
Number of obs: 120, groups:  GeneticNK, 45; FosterNK, 41

Fixed effects:
                 Estimate Std. Error      df t value Pr(>|t|)
(Intercept)       16.4744     0.8423 34.7895  19.559   <2e-16 ***
TreatmentEnlarged  0.9611     1.3973 36.4361   0.688    0.496
TreatmentReduced   1.0603     1.2371 37.9355   0.857    0.397
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6: models for testing the influence of the brood size manipulation on exploratory score. **A)** linear mixed model for testing random factors FosterNK and Genetic NK (not nested in pair) **B)** linear mixed model for testing influence of brood size manipulation on exploratory behavior.

```
lmer3: COR_LOC ~ 1 + ((1 | FosterNK) + (1 | GeneticNK))
lmer4: COR_LOC ~ Treatment + ((1 | FosterNK) + (1 | GeneticNK))
      npar    AIC    BIC  logLik deviance Chisq Df Pr(>Chisq)
lmer3    4 764.92 776.07 -378.46   756.92
lmer4    6 768.07 784.79 -378.03   756.07 0.848  2     0.6544
```

Figure 7*:* anova between LMM with treatment and without treatment.

Appendix 3 LMM Analysis of the relationship between treatment and weight of day 14

Here, full models to investigate the relationship between the influence of the treatment on the weight of the birds (figure 8) can be found. For this, the weight of day 14. An anova was carried out to test the fit of both models (figure 9).

A.

```
Linear mixed model fit by REML. t-tests use Satterthwaite's
  method [lmerModLmerTest]
Formula:
Weight_14 ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK) +
    (1 | Pair))
   Data: MydataWeight

REML criterion at convergence: 320.4

Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.98491 -0.59286  0.02697  0.53620  2.55589

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept) 0.0000   0.0000
 Pair:FosterNK  (Intercept) 0.7532   0.8679
 Pair           (Intercept) 0.2583   0.5082
 Residual                   0.7002   0.8368
Number of obs: 105, groups:
Pair:GeneticNK, 50; Pair:FosterNK, 41; Pair, 33

Fixed effects:
            Estimate Std. Error     df t value Pr(>|t|)
(Intercept)   17.151      0.188 31.542   91.24   <2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

B.

```
Linear mixed model fit by maximum likelihood . t-tests use
  Satterthwaite's method [lmerModLmerTest]
Formula:
Weight_14 ~ Treatment + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK)
 +
    (1 | Pair))
   Data: MydataWeight

     AIC      BIC   logLik deviance df.resid
   328.4    347.0   -157.2    314.4       98

Scaled residuals:
     Min       1Q   Median       3Q      Max
-2.09734 -0.62287  0.00772  0.61626  2.60771

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept) 0.0000   0.0000
 Pair:FosterNK  (Intercept) 0.5596   0.7480
 Pair           (Intercept) 0.2988   0.5466
 Residual                   0.6966   0.8346
Number of obs: 105, groups:
Pair:GeneticNK, 50; Pair:FosterNK, 41; Pair, 33

Fixed effects:
                 Estimate Std. Error      df t value Pr(>|t|)
(Intercept)       17.2679     0.2791 30.7511  61.863   <2e-16
TreatmentEnlarged -0.7736     0.4568 36.0361  -1.694    0.099
TreatmentReduced   0.1848     0.3944 36.7926   0.469    0.642
```

Figure 8: models for testing the influence of the brood size manipulation on body weight day 14.
A) linear mixed model for testing random factors FosterNK and Genetic NK nested in pair **B)** linear mixed model for testing influence of brood size manipulation on body weight day 14.

```
Models:
lmer8: Weight_14 ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK) +
lmer8:     (1 | Pair))
lmer9: Weight_14 ~ Treatment + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK) +
lmer9:     (1 | Pair))
      npar    AIC    BIC  logLik deviance  Chisq Df Pr(>Chisq)
lmer8    5 328.88 342.15 -159.44   318.88
lmer9    7 328.38 346.95 -157.19   314.38 4.5027  2     0.1053
```

***Figure 9**:* anova between LMM with treatment and without treatment.

Appendix 4 LMM Analysis of the relationship between exploratory score and body weight (before testing)

Figure 10 shows the models used for testing the relationship between exploratory score and body weight. The body weight right before testing was used. Again, an anova was performed to investigate the fit of both models (figure 11).

A.

```
Linear mixed model fit by REML. t-tests use Satterthwaite's method [
lmerModLmerTest]
Formula: COR_LOC ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
   Data: MydataWeight

REML criterion at convergence: 656.4

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.4440 -0.4406 -0.1035  0.4928  3.2864

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept)  0.0000  0.0000
 Pair:FosterNK  (Intercept)  0.7281  0.8533
 Residual                   30.1476  5.4907
Number of obs: 105, groups:  Pair:GeneticNK, 50; Pair:FosterNK, 41

Fixed effects:
            Estimate Std. Error      df t value Pr(>|t|)
(Intercept)  17.0359     0.5568 34.1144    30.6   <2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

B.

```
Formula: COR_LOC ~ Weight_before + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
   Data: MydataWeight

     AIC      BIC   logLik deviance df.resid
   663.7    676.9   -326.8    653.7      100

Scaled residuals:
    Min      1Q   Median      3Q     Max
-2.26809 -0.52430 -0.03394  0.50449  3.07188

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept)  0.3651  0.6042
 Pair:FosterNK  (Intercept)  0.6042  0.7773
 Residual                   28.6445  5.3521
Number of obs: 105, groups:  Pair:GeneticNK, 50; Pair:FosterNK, 41

Fixed effects:
             Estimate Std. Error       df t value Pr(>|t|)
(Intercept)     1.4018     8.3598 102.8621   0.168   0.8672
Weight_before   0.9262     0.4942 102.9737   1.874   0.0638 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Figure 10: models for testing the relationship between exploratory score and body weight.
A) linear mixed model for testing random factors GeneticNK and FosterNK (nested in pair) **B)** linear mixed model for testing the relationship between exploratory score and body weight.

```
Models:
lmer8: COR_LOC ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
lmer9: COR_LOC ~ Weight_before + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
      npar    AIC    BIC  logLik deviance  Chisq Df Pr(>Chisq)
lmer8    4 665.04 675.66 -328.52   657.04
lmer9    5 663.65 676.92 -326.83   653.65 3.3903  1    0.06558 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Figure 11*:* anova between LMM with weight and without weight.

Appendix 5 LMM Analysis testing relationship between age and exploratory score

Lastly, the relationship between age and exploratory score (figure 12) was investigated. Both models were again compared with each other via an anova (figure 13).

A.

```
Linear mixed model fit by REML. t-tests use Satterthwaite's method [
lmerModLmerTest]
Formula: COR_LOC ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
   Data: MydataWeight

REML criterion at convergence: 656.4

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.4440 -0.4406 -0.1035  0.4928  3.2864

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept)  0.0000  0.0000
 Pair:FosterNK  (Intercept)  0.7281  0.8533
 Residual                   30.1476  5.4907
Number of obs: 105, groups:  Pair:GeneticNK, 50; Pair:FosterNK, 41

Fixed effects:
            Estimate Std. Error      df t value Pr(>|t|)
(Intercept)  17.0359     0.5568 34.1144    30.6   <2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
convergence code: 0
```

B.

```
Linear mixed model fit by maximum likelihood . t-tests use
  Satterthwaite's method [lmerModLmerTest]
Formula: COR_LOC ~ Age + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
   Data: MydataWeight

     AIC      BIC   logLik deviance df.resid
   656.7    670.0   -323.3    646.7      100

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.5282 -0.5868 -0.0620  0.4504  3.2350

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept)  0.00000 0.0000
 Pair:FosterNK  (Intercept)  0.04274 0.2067
 Residual                   27.64794 5.2581
Number of obs: 105, groups:  Pair:GeneticNK, 50; Pair:FosterNK, 41

Fixed effects:
            Estimate Std. Error        df t value Pr(>|t|)
(Intercept) 12.56899    1.44464  96.18664   8.700 9.05e-14 ***
Age          0.05527    0.01674 104.89767   3.302  0.00131 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Figure 12: models for testing the relationship between exploratory score and age. **A)** linear mixed model for testing random factor GeneticNK **B)** linear mixed model for testing the relationship between exploratory score and age.

```
Data: MydataWeight
Models:
lmerday1: COR_LOC ~ 1 + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
lmerday2: COR_LOC ~ Age + ((1 | Pair:FosterNK) + (1 | Pair:GeneticNK))
          npar    AIC    BIC  logLik deviance  Chisq Df Pr(>Chisq)
lmerday1    4 665.04 675.66 -328.52   657.04
lmerday2    5 656.69 669.96 -323.35   646.69 10.351  1   0.001294 **
```

Figure 13*:* anova between LMM with and without age.

In addition to investigating the relationship between age and exploratory score, the interaction between treatment and age on the influence of exploratory score was also tested (figure 14 and 15). In the first model (figure 14), exploratory score as dependent variable was used, treatment and age as fixed variable and genetic and foster nest box (nested in pair) as random factors. In the second model (figure 16)), exploratory score as dependent variable was used, the interaction between treatment and age as fixed variable and genetic and foster nest box (nested in pair) as random factors.

```
Linear mixed model fit by maximum likelihood . t-tests use
  Satterthwaite's method [lmerModLmerTest]
Formula:
COR_LOC ~ Treatment + Age + (1 | Pair:FosterNK) + (1 | Pair:GeneticNK)
   Data: MydataWeight

     AIC      BIC   logLik deviance df.resid
   659.5    678.1   -322.8    645.5       98

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.5478 -0.6698 -0.0826  0.4530  3.2472

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept)  0.00    0.000
 Pair:FosterNK  (Intercept)  0.00    0.000
 Residual                   27.38    5.233
Number of obs: 105, groups:
Pair:GeneticNK, 50; Pair:FosterNK, 41

Fixed effects:
                  Estimate Std. Error        df t value Pr(>|t|)
(Intercept)        12.05726    1.54720 105.00000   7.793 4.96e-12
TreatmentEnlarged   0.63404    1.32840 105.00000   0.477  0.63414
TreatmentReduced    1.26888    1.16921 105.00000   1.085  0.28030
Age                 0.05428    0.01670 105.00000   3.250  0.00155

(Intercept)       ***
TreatmentEnlarged
TreatmentReduced
Age               **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) TrtmnE TrtmnR
TrtmntEnlrg -0.327
TretmntRdcd -0.294  0.399
Age         -0.860  0.028 -0.058
```

Figure 14: LMM with LOCOM as dependent variable, treatment and age (day) as fixed and Genetic nest box as random factor.

```
Linear mixed model fit by maximum likelihood . t-tests use
  Satterthwaite's method [lmerModLmerTest]
Formula:
COR_LOC ~ Treatment * Age + (1 | Pair:FosterNK) + (1 | Pair:GeneticNK)
   Data: MydataWeight

     AIC      BIC   logLik deviance df.resid
   652.6    676.5   -317.3    634.6       96

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.7404 -0.7026 -0.0130  0.5050  2.7938

Random effects:
 Groups         Name        Variance Std.Dev.
 Pair:GeneticNK (Intercept)  0.0000  0.0000
 Pair:FosterNK  (Intercept)  0.1519  0.3897
 Residual                   24.5332  4.9531
Number of obs: 105, groups:
Pair:GeneticNK, 50; Pair:FosterNK, 41

Fixed effects:
                      Estimate Std. Error        df t value
(Intercept)           11.26510    1.80487  95.78144   6.242
TreatmentEnlarged     -9.32132    4.57837 101.62872  -2.036
TreatmentReduced       6.99638    3.09049  87.75795   2.264
```

```
Age                       0.06427    0.02056 104.82282   3.126
TreatmentEnlarged:Age     0.12872    0.05652  90.73884   2.277
TreatmentReduced:Age     -0.06896    0.03494 104.43613  -1.974
                        Pr(>|t|)
(Intercept)             1.18e-08 ***
TreatmentEnlarged        0.04436 *
TreatmentReduced         0.02605 *
Age                      0.00229 **
TreatmentEnlarged:Age    0.02512 *
TreatmentReduced:Age     0.05106 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) TrtmnE TrtmnR Age    TrtE:A
TrtmntEnlrg -0.394
TretmntRdcd -0.584  0.230
Age         -0.909  0.358  0.531
TrtmntEnl:A  0.330 -0.961 -0.193 -0.364
TrtmntRdc:A  0.535 -0.211 -0.932 -0.588  0.214
convergence code: 0
```

Figure 15: LMM with LOCOM as dependent, interaction between treatment and age as fixed and genetic nest box as random

Both models were compared with an anova to see investigate which model fit the data the best. The comparison between the models showed a significant result (Anova, df = 2, x^2 (2) = 10.896, p = 0.0043; figure 16), which confirms that there is indeed an interaction between the treatment and age. In figure 17 the exploratory score is visualized grouped per treatment.

```
Data: MydataWeight
Models:
lmerday14: COR_LOC ~ Treatment + Age + (1 | Pair:FosterNK) + (1 | Pair:Gene
ticNK)
lmerday15: COR_LOC ~ Treatment * Age + (1 | Pair:FosterNK) + (1 | Pair:Gene
ticNK)
          npar    AIC    BIC  logLik deviance  Chisq Df Pr(>Chisq)
lmerday14    7 659.52 678.10 -322.76   645.52
lmerday15    9 652.62 676.51 -317.31   634.62 10.896  2   0.004306 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Figure 16: anova between model with interaction between age and treatment and without.

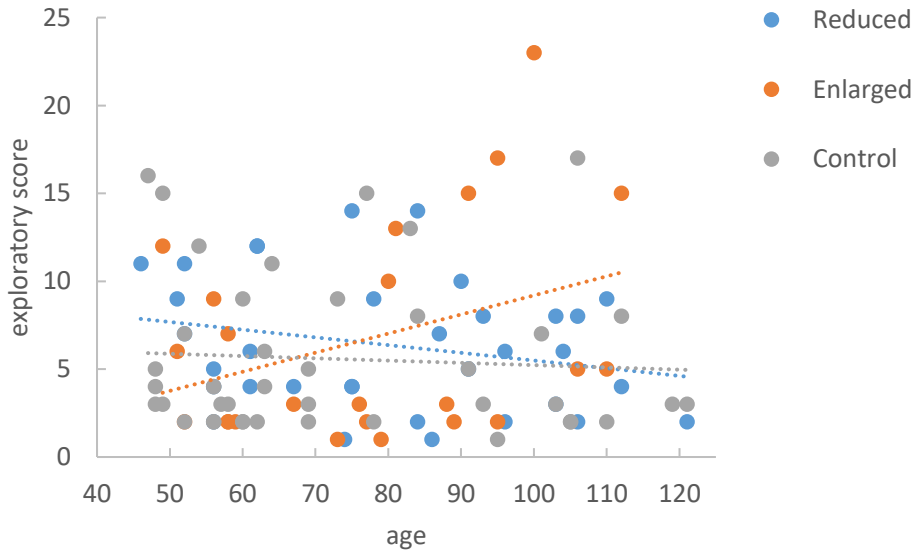

Figure 17: correlation between exploratory score and age. Sorted per treatment (control, reduced and enlarged).

The interaction between the fixed factor (treatment) and covariate (age) was also investigated. For this, a model was constructed where exploratory score was the dependent variable, treatment (control, reduced and enlarged) was fixed and age was a covariate.

Model:

age.lm <-lm(LOCOM~Age*Treatment, data= MydataWeight)

Subsequently, the slopes were estimated and compared with each other. No significant difference between the three slopes has been found (see figure 18 and 20)

```
> emtrends(age.lm, pairwise~Treatment, var = "Age")
$emtrends
 Treatment Age.trend     SE df lower.CL upper.CL
 Control      0.0384 0.0192 99  0.00025   0.0765
 Enlarged     0.1086 0.0493 99  0.01083   0.2064
 Reduced     -0.0190 0.0264 99 -0.07133   0.0333

Confidence level used: 0.95

$contrasts
 contrast           estimate     SE df t.ratio p.value
 Control - Enlarged  -0.0703 0.0529 99 -1.328  0.3830
 Control - Reduced    0.0574 0.0326 99  1.759  0.1889
 Enlarged - Reduced   0.1276 0.0559 99  2.283  0.0628

P value adjustment: tukey method for comparing a family of 3 estimates
```

Figure 18: comparison of the estimated slopes between control, reduced and enlarged.

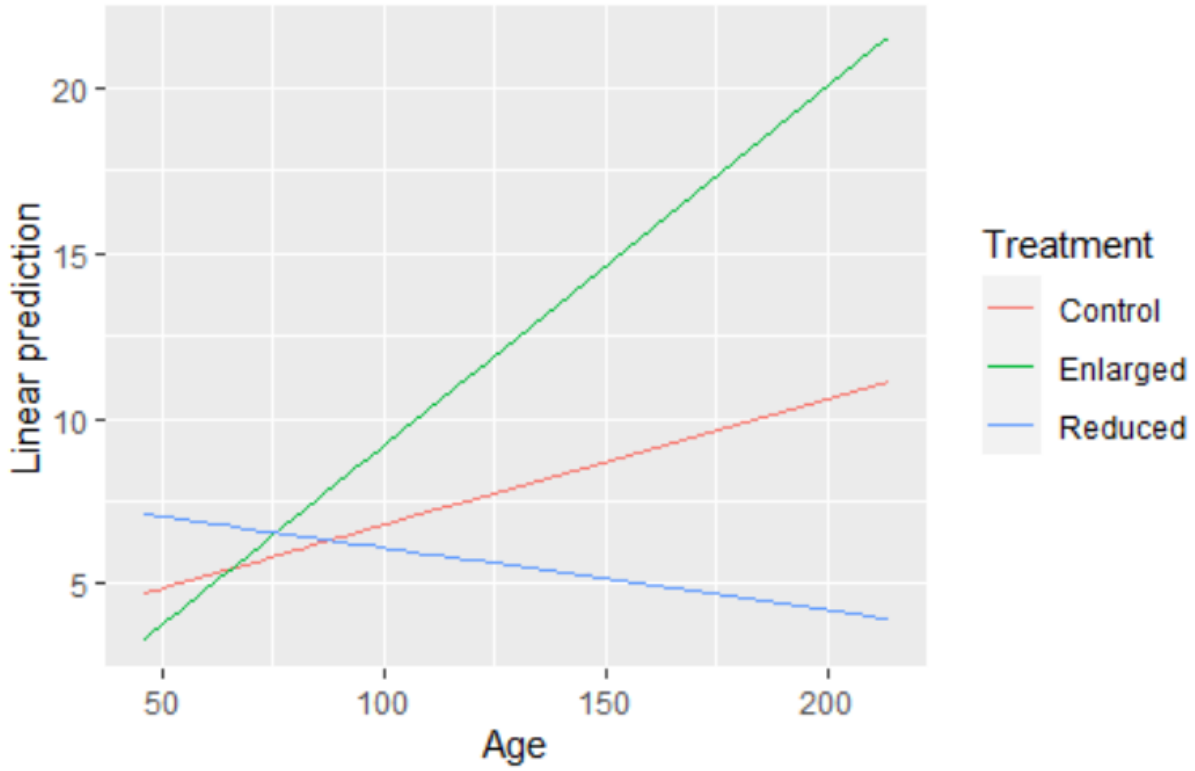

***Figure 19*:** predictions of the slopes of the three different groups (control, enlarged and reduced).

Relationship between age and exploratory score (without correction for date)

The relationship between age (without correction for date) and exploratory behavior of the great tits was analysed. The first model (to test for the random structure) showed that 35.07% of the variance can be explained by an effect of origin and 64.93% is residual variance (table 1).

Table 1: LMM results model 7. n = 120 (genetic n = 56, foster n = 46). Signif. codes: 0 '***'

fixed effect	**estimate**	**std. error**	**df**	**t value**	**p-value**	**variance**	**std. dev.**
intercept	6.18	0.55	51.52	11.16	$<2.33e^{-15***}$	-	-
random effect							
origin	-	-	-	-	-	7.54	2.75
rearing	-	-	-	-	-	0.00	0.00
residual	-	-	-	-	-	13.96	3.74

The second model showed no significant relationship between age and exploratory score (LMM, p = 0.082; table 2).

Table 2: LMM results model 8. n = 120 (genetic n = 56, foster n = 46). Signif. code: 0 '***'

fixed effect	**estimate**	**std. error**	**df**	**t value**	**p-value**	**variance**	**std. dev.**
age	0.023	0.014	98.32	1.70	0.092	-	-
intercept	4.27	1.24	104.94	3.43	$8.53e^{-4}$		
random effect							
origin	-	-	-	-	-	7.08	2.66
rearing	-	-	-	-	-	0.00	0.00
residual	-	-	-	-	-	14.11	3.76

A comparison between both models showed no significant difference (Anova, df = 1, x^2 (1) = 2.86, p = 0.091). In figure 20, a graph was made to visualize the relationship between age and exploratory behavior.

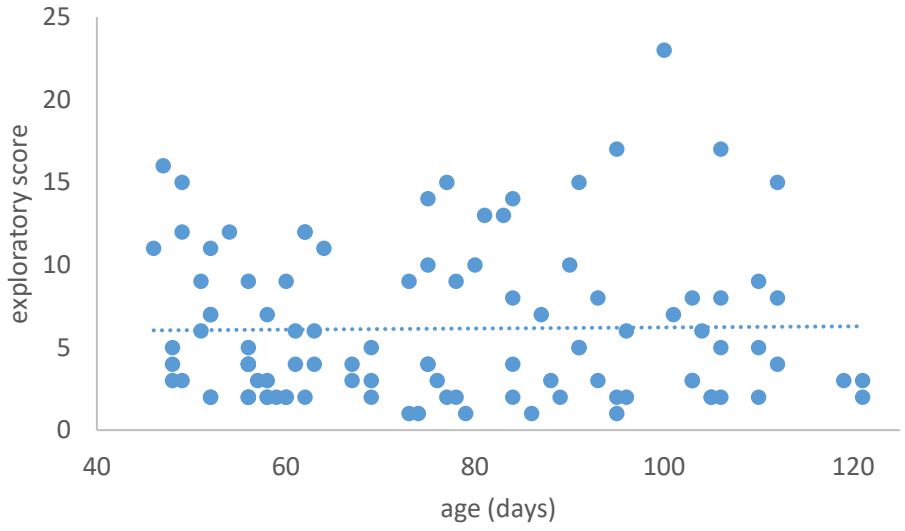

Figure 20: graph of the exploratory score and age of the birds (testing date – hatching date) (n=120).

Appendix 6 References

Auclair, G., and Weber, M. (2012). Mechanisms of DNA methylation and demethylation in mammals. *Biochimie*, *94*(11), 2202–2211. https://doi.org/10.1016/j.biochi.2012.05.016

Bossdorf, O., Richards, C. L., & Pigliucci, M. (2008). Epigenetics for ecologists. *Ecology Letters*. https://doi.org/10.1111/j.1461-0248.2007.01130.x

Fidler, A. E., Van Oers, K., Drent, P. J., Kuhn, S., Mueller, J. C., & Kempenaers, B. (2007). Drd4 gene polymorphisms are associated with personality variation in a passerine bird. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1619), 1685–1691. https://doi.org/10.1098/rspb.2007.0337

van Gurp, T. P., Wagemaker, N. C. A. M., Wouters, B., Vergeer, P., Ouborg, J. N. J., & Verhoeven, K. J. F. (2016). EpiGBS: Reference-free reduced representation bisulfite sequencing. *Nature Methods*, *13*(4), 322–324. https://doi.org/10.1038/nmeth.3763

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nature Genetics*. https://doi.org/10.1038/ng1089

Korsten, P., Mueller, J. C., Hermannstädter, C., Bouwman, K. M., Dingemanse, N. J., Drent, P. J., Liedvogel, M., Matthysen, E., Van Oers, K., Van Overveld, T., Patrick, S. C., Quinn, J. L., Sheldon, B. C., Tinbergen, J. M., & Kempenaers, B. (2010). Association between DRD4 gene polymorphism and personality variation in great tits: A test across four wild populations. *Molecular Ecology*, *19*(4), 832–843. https://doi.org/10.1111/j.1365-294X.2009.04518.x

Kvist, L., Martens, J., Higuchi, H., Nazarenko, A. A., Valchuk, O. P., & Orell, M. (2003). Evolution and genetic structure of the great tit (Parus major) complex. *Proceedings of the Royal Society B: Biological Sciences*, *270*(1523), 1447–1454. https://doi.org/10.1098/rspb.2002.2321

Ledón-Rettig, C. C., Richards, C. L., & Martin, L. B. (2013). Epigenetics for behavioral ecologists. In *Behavioral Ecology* (Vol. 24, Issue 2, pp. 311–324). https://doi.org/10.1093/beheco/ars145

Munafò, M. R., Yalcin, B., Willis-Owen, S. A., & Flint, J. (2008). Association of the Dopamine D4 Receptor (DRD4) Gene and Approach-Related Personality Traits: Meta-Analysis and New Data. *Biological Psychiatry*, *63*(2), 197–206. https://doi.org/10.1016/j.biopsych.2007.04.006

van Oers, K., De Jong, G., Drent, P. J., and Van Noordwijk, A. J. (2004). A genetic analysis of avian personality traits: Correlated, response to artificial selection. *Behavior Genetics*, *34*(6), 611–619. https://doi.org/10.1007/s10519-004-5588-z

Qian, A., Tao, J., Wang, X., Liu, H., Ji, L., Yang, C., Ye, Q., Chen, C., Li, J., Cheng, J., Wang, M., & Zhao, K. (2018). Effects of the 2-repeat allele of the DRD4 gene on neural networks associated with the prefrontal cortex in children with ADHD. *Frontiers in Human Neuroscience*, *12*. https://doi.org/10.3389/fnhum.2018.00279

Relyea, R. A. (2005). Phenotypic plasticity in the interactions and evolution of species. *Ecology*, *86*(7), 321–326. https://doi.org/10.2307/3669131

Richards, E.J. *(*2006*).* Inherited epigenetic variation – revisiting soft inheritance. *Nat. Rev. Genet*., 7, 395–401

Riyahi, S., Sánchez-Delgado, M., Calafell, F., Monk, D., & Senar, J. C. (2015). Combined epigenetic and intraspecific variation of the DRD4 and SERT genes influence novelty seeking behavior in great tit Parus major. *Epigenetics*, *10*(6), 516–525. https://doi.org/10.1080/15592294.2015.1046027

Rodenburg, T. B., & van Oers, K. (2010). Interactions between Behavior and Genetics in Wild and Domestic Bird Populations. *Proceedings of the 9th World Congress on Genetic*

Applied to Livestock Production (WCGALP), Leipzig, Germany, 1-6 August 2010, 99. Retrieved from http://edepot.wur.nl/161389

Sheldon, E. L., Schrey, A. W., Ragsdale, A. K., & Griffith, S. C. (2018). Brood size influences patterns of DNA methylation in wild Zebra Finches (Taeniopygia guttata). *The Auk*, *135*(4), 1113–1122. https://doi.org/10.1642/auk-18-61.1

Sepers, B., van den Heuvel, K., Lindner, M., Viitaniemi, H., Husby, A., and van Oers, K. (2019). Avian ecological epigenetics: pitfalls and promises. *Journal of Ornithology*, *160*(4), 1183–1203. https://doi.org/10.1007/s10336-019-01684-5

Verhoeven, K. J. F., VonHoldt, B. M., & Sork, V. L. (2016). Epigenetics in ecology and evolution: What we know and what we need to know. *Molecular Ecology*, *25*(8), 1631–1638. https://doi.org/10.1111/mec.13617

Verhulst, E. C., Mateman, A. C., Zwier, M. V., Caro, S. P., Verhoeven, K. J. F., & Van Oers, K. (2016). Evidence from pyrosequencing indicates that natural variation in animal personality is associated with DRD4 DNA methylation. *Molecular Ecology*, *25*(8), 1801–1811. https://doi.org/10.1111/mec.13519

Wolff, G. L., Kodell, R. L., Moore, S. R., & Cooney, C. A. (1998). *Maternal epigenetics and methyl supplements affect agouti gene expression in A vy /a mice*. Retrieved from www.fasebj.org

Zimmer, C., Boogert, N. J., & Spencer, K. A. (2013). Developmental programming: Cumulative effects of increased pre-hatching corticosterone levels and post-hatching unpredictable food availability on physiology and behavior in adulthood. *Hormones and Behavior*, *64*(3), 494–500. https://doi.org/10.1016/j.yhbeh.2013.07.002