Investigating the Heritability of Egg Traits in Blue Tits (*Cyanistes caeruleus*)

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Like most members of the family Paridae blue tits lay white eggs speckled with reddish brown spots. Patterns of these spots vary in every egg, over the period several hypotheses have been proposed to understand the reasoning behind these spots, however very little have been known about mode of heritability and genetic architecture of these maculation spots. In an order to understand genetics behind these spots we studied wild population of blue tits in southwestern Finland. Firstly, we did a scoring test for three traits (distribution, size and intensity) of maculation spots. Then we did variance partitioning of all three maculation traits along with the mass of eggs while examining the effect of age and tarsus size of each female and found individual heritability estimate of each trait. We found out that most of these traits were heritable and we also found out that younger females were laying bigger eggs. In the end by using multivariate model, we found out the genetic correlation between three maculation traits to see if they can evolve independently. We found out that size and intensity of spots have positive correlation, eggs with more intense spots also tend to have bigger sized spots on the other hand distribution of spots didn't show any such relationship. Overall, our results contribute to understanding the evolution of avian eggshell, however further improvements are necessary in an order to understand the genetic architecture of eggshell spottiness better.

Key words: blue tits, pigmentation, maculation, heritability, animal models

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1 Introduction

The diversity of eggshell colour and patterning in birds has intrigued researchers for over a century. From pure white to richly speckled and vividly coloured eggs, the variation is both visually striking and biologically significant. These traits are thought to serve a range of adaptive functions, from camouflage to communication. This introduction begins by outlining the main hypotheses explaining the evolution of eggshell maculation, then moves to the genetic underpinnings of these traits. Finally, it frames the rationale and objectives of this study on blue tits (*Cyanistes caeruleus*), a widely used model species in avian biology.

1.1 Hypothesis Behind Eggshell Maculation

Many avian species, particularly within the order Passeriformes, lay eggs with distinct pigment patterns known as maculation i.e. red, brown, or black protoporphyrin spots speckled on a white eggshell (Higham & Gosler, 2006; Attard, Bowen, & Portugal, 2023). These spots vary widely in size, intensity, and distribution, both across and within species, and even among eggs laid by the same female. Several hypotheses have been proposed to explain the adaptive functions of this remarkable diversity. These explanations generally fall into two broad categories: those that focus on immediate environmental causes and those that suggest longer-term evolutionary or signalling functions. An example of such hypothesis is the camouflage hypothesis, which suggests that speckled pigmentation enables concealing the eggs, which reduces the risk of predation. On the contrary, the structural integrity hypothesis proposes that maculation is a physiological adaptation that balances thinner areas of the eggshell and thus reinforces mechanical resistance. These are primarily responses to environmental pressures.

In contrast, other hypotheses such as sexually selected signalling propose that maculation serves as a visual cue of female quality or egg viability, potentially influencing male behaviour. Likewise, maternal investment signalling links the extent of pigmentation to hormone levels or immune function, offering insight into maternal condition. These perspectives reflect more evolutionary and communicative interpretations of maculation.

Additional functions include thermoregulation, where pigmentation may reduce water loss in arid environments, and ultraviolet (UV) protection, in which the spots shield developing embryos from harmful radiation. The egg recognition hypothesis is also notable, especially in host species facing brood parasitism, where maculation aids in distinguishing own eggs from those of parasitic birds.

While Table 1 provides a summary of seven prominent hypotheses, it is not exhaustive. Other mechanisms may also contribute to the observed variation, and many of the proposed functions are not mutually exclusive. In fact, multiple selective pressures may act simultaneously, shaping the expression of maculation traits in complex ways.

Table 1 overview of some hypothesis for variation in maculation spots in birds, for each hypothesis their explanation is give along with the source, hypothesis is numbered from 1 to 7.

ID	Hypothesis	Explanation	Source
1	Camouflage	help the eggs to blend in with environment better to hide from predators	(Lack, 1958)
2	Structural Integrity	compensates the reduced eggshell thickness	(Gosler et.al., 2005)
3	Sexually Selected Signal	used by females to send visual signals to mates of quality of female and/or health of egg	(Moreno & Osorno, 2023, Cherry & Gosler, 2010)
4	Thermoregulation	help preventing the loss of moisture and protect eggs from dry environment	(Bakken *et al.* 1978)
5	UV Protection	protects the developing embryo from UV radiations	Orłowski, Niedzielski, Merta, & et al., 2020)
6	Maternal Investment	reflect investment of mother, hormone levels or immune functions	(Holveck et al., 2012)
7	Egg Recognition	affects the ability of host to reject parasitic eggs	(Fernandez-Duque et al., 2025)

1.2 Genetic Architecture of Eggshell Maculation

The hypotheses outlined above, such as those related to camouflage, sexual signalling, and maternal investment, all rest on the assumption that variation in eggshell maculation can be acted upon by natural or sexual selection. For selection to produce evolutionary change, the traits in question must not only vary among individuals but also be at least partly genetically inherited. Therefore, understanding the heritability of maculation patterns is essential for assessing their evolutionary potential. If these traits are under genetic control, they may evolve over generations in response to selective pressures. The patterns of maculation are variably determined by genetic control, environmental variability, or development noise, and this difference is critical in explaining their role in long-term adaptation.

Heritability in this context refers to the ratio of the entire phenotypic variance explained by the additive genetic variance, which is the part of the genetic variance that is passed by the parents to the offspring and is influenced by selection. The parent offspring resemblance is based on additive genetic variance (absence of dominance and epistasis) and has a greater evolutionary potential since selection can better regulate phenotypic frequencies between generations.

Birds especially have complex genetic structures due to their sex-determination mechanism. The heterogametic sex is female (ZW) and homogametic sex is male (ZZ). Sex-linked inheritance arises when genes that are found on the Z or W chromosomes are related to traits. Notably, Fossvoy et al. (2016) have hypothesised that a gene affecting eggshell colour is on the W chromosome, and is passed down exclusively by mums to daughters, and thus serves as an example of maternal inheritance. The effects of sex on maculation traits in great tits (*Parus major*) have also been recorded by Gosler et al. (2005) which points out the complexity of the genetic factors that determine such traits. Collectively, the results make it clear that the non-Mendelian aspects of sex linkage and maternal effects cannot be ignored when interpreting heritability estimates.

There is a significant literature on the evolutionary adaptations in great tits. An example of this is the argument by Gosler et al. (2001) that pigmentation spots reinforce weak parts of the eggshell, thus providing structural benefits. These results raised questions about whether such traits are condition-dependent or genetically controlled. Further research in blue tits has extended this direction of research. In 2021, Cribellier observed that some of the maculation characteristics serve as visual cues to female state or reproductive investment. However, the extent to which these characteristics are inheritable and genetically limited has remained elusive, with an open question remaining, do these cues have stability and are passed down through generations, or are they largely mediated by short-term environmental fluctuation?

1.3 Why Heritability Estimation Matters

Eggshell maculation has been cited as having adaptive functions in camouflage, structural reinforcement, intraspecific communication and maternal investment indicators; however, the degree to which these phenotypes are genetically determined has been inadequately explained. Assessing their heritability is essential to understanding whether maculation can respond to natural or sexual selection and thus evolve over generations.

Evolutionary quantitative genetics focuses on the study of heritability, which is the relationship between the total phenotypic variance in a trait and additive genetic variance. This is typically expressed as

$$h^2 = \frac{V_A}{V_P} \tag{1}$$

where h^2 is the narrow-sense heritability, V_A is the additive genetic variance, and V_P is the total phenotypic variance. A high heritability value suggests that much of the trait variation among individuals is due to inherited genetic differences, making the trait more responsive to selection. In contrast, low heritability implies that environmental effects or non-additive genetic components dominate, reducing the potential for evolutionary change.

In addition to heritability assessment, it is necessary to quantify genetic correlations among traits. Genetic correlation is a measure of the degree of the common genetic basis of two traits and is not just the phenotypic correlation. It is typically defined as:

$$r_G = \frac{Cov_G(X,Y)}{\sqrt{V_{G,X} \cdot V_{G,Y}}} \tag{2}$$

where $Cov_G(X, Y)$ is the additive genetic covariance between traits X and Y, and $V_{G,X}$, $V_{G,Y}$ are their respective additive genetic variances.

These correlations help us to understand evolutionary constraints and trade-offs. For example, if two traits are strongly genetically correlated, then selection on one trait may inadvertently lead to changes in the other, even if that change is maladaptive. This means traits cannot always evolve independently, and their evolutionary trajectories may be constrained by underlying genetic architecture.

The characterization of maculation features such as size of spot, spot intensity and spot distribution is especially important as these features are postulated to be adaptively advantageous in photoreception, camouflage and social signaling. It is thus necessary to find out whether these characteristics are genetically connected; this connection can be indicative of pleiotropy or linkage disequilibrium. Quantitative genetic analyses can be used to determine the effect of selection on one trait (such as spot intensity as a signal) on other traits (such as spot size), and whether there is a possibility of coevolution of traits. Modern statistical practices, and in particular animal models used in a mixed-effects context, have been highly effective in the estimation of heritability and genetic correlations. These models allow a powerful analysis of the variation in wild populations with known pedigrees by

decomposing the phenotypic variance into additive genetic, maternal, environmental and residual components, thus increasing statistical power and allowing traits to be traced over generations.
The current study established heritability of maculation patterns and egg mass in blue tits, thus identifying whether these characteristics have adequate additive genetic variance to allow them to evolve under selection. Further genetic correlation analyses clarified whether the features of maculation and reproductive investment strategies are phenotypically modulated independently or are a genetically unitary entity.

1.4 Study Species and Trait Focus

The current study focuses on a relatively small species of insectivorous passerine, blue tit, that is a member of the family Paridae, which encloses also for example great tits, coal tits, and marsh tits. Blue tits are found across Europe and some areas in western Asia and are especially common in temperate woodlands and urban environments. This ease of breeding in man-made nest boxes and relatively high reproductive output has made them a model animal in avian behavioural ecology, life-history evolution and quantitative genetics (Charmantier et al., 2015).

The eggs of blue tit clutches are white with reddish-brown macules mainly found at the broad end of each egg. The size, intensity and distribution of these markings show a lot of individual and clutch-to-clutch variation, making them suitable to a quantitative analysis of traits. In addition to the aesthetic value of such variability, it can give clues to the underlying physiological or genetic processes. The family Paridae has long been the object of classic studies of eggshell characteristics, especially in the great tit. As an example, Gosler et al. (2005) hypothesised that pigmentation patterns have structural or adaptive roles with regard to the size of an eggshell. The family Paridae has long been the object of classic studies on eggshell characteristics, especially in the great tit. For instance, Gosler et al. (2005) suggested that pigmentation patterns might have structural or adaptive significance, particularly in relation to eggshell thickness. Moreover, studies in great tits have explored both environmental and genetic influences on maculation traits, including evidence for sex-linked inheritance and potential heritability. These findings provide a valuable foundation for understanding how such traits may evolve under selection.

However, despite this growing body of work in great tits, the genetic basis of maculation traits remains comparatively underexplored in blue tits. This gap is particularly noteworthy given the extensive use of blue tits in behavioural and ecological research. Their striking maculation patterns and known maternal variation make them a compelling system for addressing unresolved questions about genetic architecture and evolutionary potential.

The current study builds on the understanding of maculation commonly framed in the parental-investment framework by adding a second, complementary measure of reproductive output, egg mass. The number of eggs regulate the rate of embryonic development, the health state of hatchlings, and the survival of offspring, in addition to heritability in some avian lineages (e.g., Kvalnes et al., 2013). This fact makes egg mass an aspect that can be subjected to natural selection hence promoting the comparative and the evolutionary views. The connection of heritability of egg mass with heritability of maculation provides a source of information on how mothers allocate resources and whether these decisions are genetically structured.

In summary, the blue tit, a species that bears the reputation of having one of the most ecologically tractable and conspicuous egg markings, offers a specially illustrative example to learn about the interaction between genetics, observation of trait expression, and the evolutionary potential.

1.5 Study Objectives

The primary aim of this study is to investigate the genetic architecture of two important egg traits in blue tits: (i) eggshell maculation: specifically, the intensity, distribution, and size of pigment spots, and (ii) egg mass: which is widely used as a proxy for maternal investment and offspring provisioning. Both sets of traits are relevant to questions of adaptation, communication, and reproductive strategy, yet their underlying genetic basis remains insufficiently understood. This study builds on previous work by Cribellier (2021), who explored phenotypic variation in maculation traits. In this study, we expand on the dataset originally analysed by Cribellier (2021) by incorporating two additional breeding seasons (2021 and 2022), resulting in a more comprehensive multi-year dataset. This increases both the sample size and temporal depth of the analysis, improving the precision of heritability estimates and enhancing our ability to detect underlying genetic signals with greater statistical power.

To estimate the heritability of individual traits, we employ univariate animal models that allow partitioning of phenotypic variance into genetic and non-genetic components. For examining whether different maculation traits share a common genetic basis, we use multivariate models to estimate genetic correlations between traits such as spot size, distribution, and intensity.

Based on prior findings in blue tits and related species, we hypothesize that egg mass will show moderate to high heritability (approximately $h^2 = 0.3 - 0.6$), consistent with previous findings in other passerines and its established role as a genetically influenced component of maternal investment. We also expect maculation traits to display some level of heritability, although environmental and condition-dependent factors may contribute to the observed variation. Finally, we anticipate that maculation traits will be positively genetically correlated, which would suggest that they are influenced by shared genetic pathways and could co-evolve under selection.

2 Materials and methods:

2.1 Study Area and Period

Data used in this study was collected from a deciduous forest area in Tammisaari, Finland (60°01′ N, 23°31′ E, altitude 24 m). This study site is about 10 km² and it is mostly surrounded by agricultural land and forest. The site in total has supported about 350 wooden nest boxes. This population was established in 2003 with an extension of box network in 2005. Data that is used in this study is the extension of data used in previous studies (Cribellier J. 2021), where author used data from year 2008 to 2020 and in this study, I added data from year 2021and 2022 and conducted the studies further.

2.2 Study Specie

Blue tits are small and colourful songbirds which are mostly native to Europe and western Asia. The start of Blue Tit breeding season varies little across its Western Palearctic range, but nesting usually begins after one month of the northern spring equinox, when the hour of daylight equals those of night. As daylight hours begin to exceed those of night, the adult Blue Tit's energies have focused on reproduction (Stenning, 2018). Blue tits have the largest clutch sizes of all birds (up-to 16 eggs); however, the majority of clutches contain 8-12 eggs, the eggs are smooth, glossy and white with patches of reddish-brown spots of different denticities typically 16mm by 12mm by size (McCambridge 2024). Female lay one egg each day for as long as it takes to build her clutch (Stenning, 2018). Blue Tits breed in secondary cavities either natural or one evacuated by other species however our study site contains nest boxes (Figure 1) which is used by these blue tits as secondary cavities these boxes allow easy collection of sufficient data along with the protection from predators.

Figure 1 wooden nest box used by blue tits as secondary cavity to lay eggs in our study field.

2.3 Data Collection

2.3.1 Egg Measurement

During breeding seasons, to monitor egg laying checks were conducted weekly of all nest boxes along with the clutch size which was recorded during entire period. Based on assumption that females lay one egg a day the laying date of first egg in incomplete clutch was back calculated. Pictures were captured in a standardized method (explained in detail later). Final clutch sizes were determined only at the onset of incubation. Incubation started with second to last egg and last approximately 14 days (Temizyürek et al. 2024). According to the hatching date was predicted and calculated and later was confirmed during daily nest checks starting from a day before expected date. Identification of species was based on visual confirmation of incubating females. Adult females were caught between 5 to 9 days post-hatching and were ringed for individual identification. Age of individual was determined by their feather coloration, we established the age as either yearling (hatched during the previous breeding season) or older (≥ 2years old) based on the color of the coverts of their primary feathers (Svensson, 1984). Morphometric measures were taken on adult birds including tarsus length, using a sliding caliper (accuracy, 0.1 mm) and body mass, using a 20g Pesola Spring balance (accuracy, 0.1g) before releasing them (Nord & Nilsson 2011).

2.3.2 Egg Photography

Throughout the breeding seasons from year 2008 to 2022 photographs of eggs were taken as a part of broader investigation. Images were taken using different highly precise cameras combination of Nikon, Canon, and Lumix to enough that documentation is of high quality. For single brood two eggs were selected randomly and photographed and left and right egg (Figure 2). This systematized method allowed us for image comparison across broods and years quiet consistently, which contributed to authentic consequent morphological and pigmentation analyses

Figure 2 An example of picture taken for one random brood containing two randomly selected eggs (left-egg and right-egg)

2.3.3 Egg Scoring

Each egg was assessed visually for three principal components pigmentation intensity of spots, distribution of spots and size of spots. The intensity of pigment is scored in 0.5 accessing from 1 for palest spots to 5 for the darkest, distribution is scored in 0.5 accessing from 1 for > 90% of spots concentrated at one end, to 5 for an equal distribution of spots across entire egg and size of spots scored in 0.5 increment from 1 for small spots to 3 for large spots Gosler et al. (2000 and 2005).

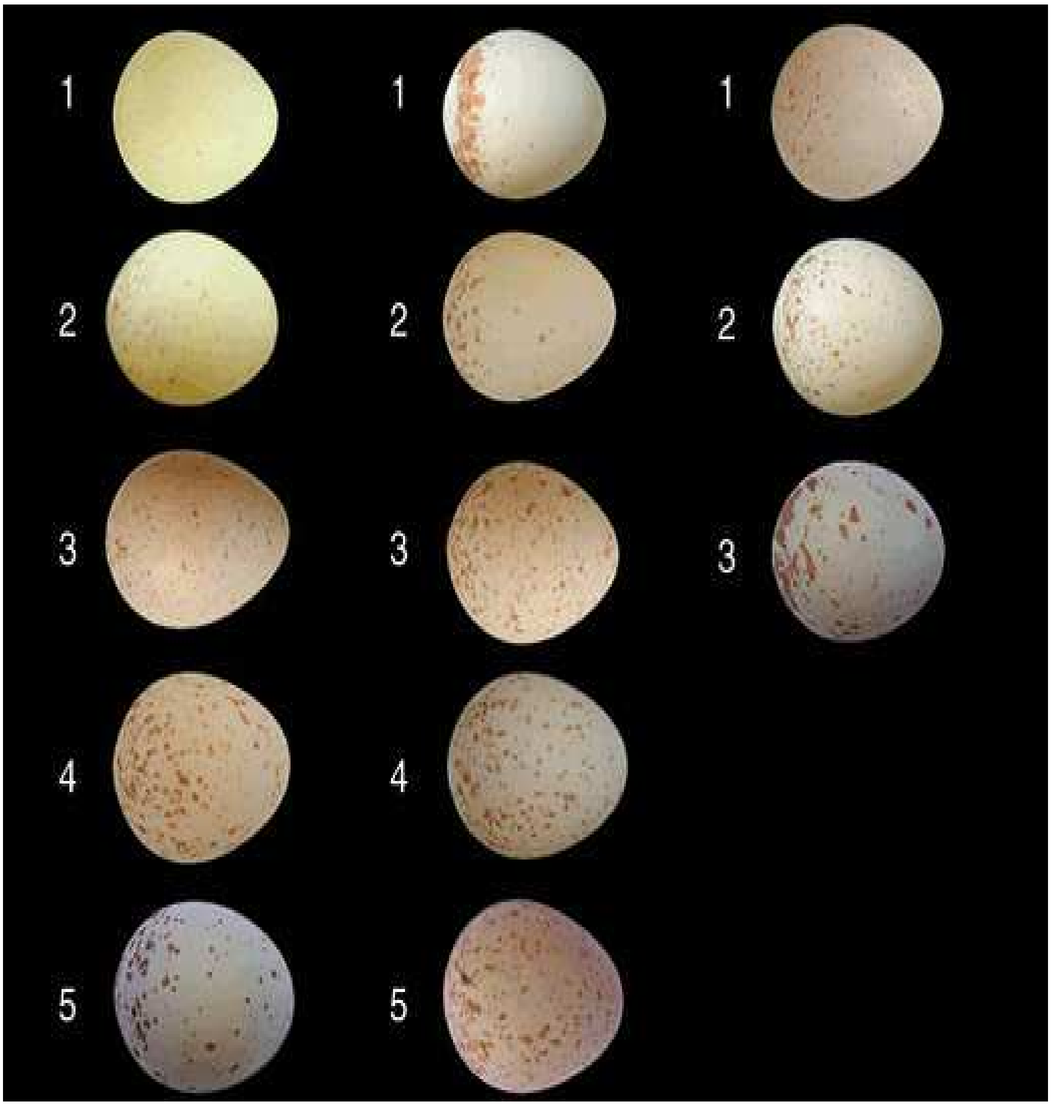

Figure 3 Egg spottiness variation among clutches of blue tits breeding in southwestern Finland. From left to right columns represent spot intensity (1-5), spot distribution (1-5) and size of spots (1-3). Rows display increasing values from top to bottom. This picture is from Sanz et al. 2009

2.4 Data preparation

2.4.1 Egg Maculation Data

We manually compiled our egg maculation data file based on the field data that was collected through standardized data entry forms. Our dataset includes detailed scoring of egg maculation traits: intensity, distribution and size. Each entry is linked to data such as year of collection, broodID, ring number, female ID. The age of egg was classified as either “y” (1 year old) or “o” (older than 1 year). Moreover, measurements of tarsus length were recorded for each female; while two separate tarsus readings (Tarsus 1 and Tarsus 2) were collected, we mainly used average of these two values for our analysis.

2.4.2 Pedigree File

The pedigree file was then prepared and structured to support our genetic analysis. Each entry in this file has unique individual ID along with its corresponding dam (mother) and sire (father) identifiers. This structure allows us to construct family trees and evaluate the patterns of heritability which make it a lot easier to link parents to their offsprings.

2.5 Statistical Analysis

All statistical analysis in my thesis are done in R version (2025.05.0+496), using base R functions along with different packages that includes Sommer (Covarrubias-Pazaran G. 2016), pedigreemm, lme4, ggplot2, dplyr and writexl. The analyses conducted are presented in more detail in the following three sections.

2.5.1 Descriptive Analysis

The initial analysis included computing statistics for egg mass and maculation traits (size, distribution and intensity), which were then visualized by generating histograms and box plots. Repeat measures per female were summarized to find within and between individual variation in egg mass. To examine repeatability and measurement consistency between left and right egg maculation traits Pearson's correlation tests were done.

2.5.2 Univariate Mixed Models and Variance Partitioning

To calibrate the sources of phenotypic variation in egg mass and maculation traits, univariate animal model with function "mmer" from Sommer package was used. Each trait was modelled with fixed effects (Age and Tarsus length) and random effect for additive genetic variance (Va), permanent environment variance (Vpe), year, and nest box identity. For estimating Va, we used statistics from known relatedness among individuals, which allowed us to partition the phenotypic variance into genetic and non-genetic components. Heritability was calculated by using following formula:

$$h^2 = \frac{Va}{Va+Vpe+Vpe+Vbox+Vyear} \tag{3}$$

Where Va is additive genetic variance, Vpe is our permanent environment, Vbox is variance due to nest box Vyear is variation from year-to-year Ve is residual variance.

Afterwards we used two-tailed Z test for determining the statistical significance of each component separately with following formula:

$$P = 2 \times (1 - \Phi(| Z |)) \tag{4}$$

Here Z = Variance component/Standard Error (SE), $\Phi(| Z |)$ is cumulative distribution function (CDF) of standard normal distribution which here is evaluated as $| Z |$.

Individual models were used for egg mass and each maculation trait (intensity, distribution and size). Box plots and pie charts were generated to visualize the variance components for each trait.

2.5.3 Multivariate Mixed Models and Genetic Correlation

For estimating genetic correlation among egg maculation traits and to access their potential for independent evolution we used a multivariate linear mixed model (Bonat 2017) using the "mmer()" function from "Sommer" R package. The response matrix included spot intensity, distribution and size which were modelled together for their potential genetic and environmental correlation and covariance. Fixed effects again included Age and Tarsus length, and random effects included additive genetic effects using pedigree-based relationship matrix, box effects, and year effects. For modelling residual variance, we used unstructured covariance matrix across traits.

3 Results

3.1 Descriptive Analysis

3.1.1 Statistics for Maculation Traits and Egg Mass

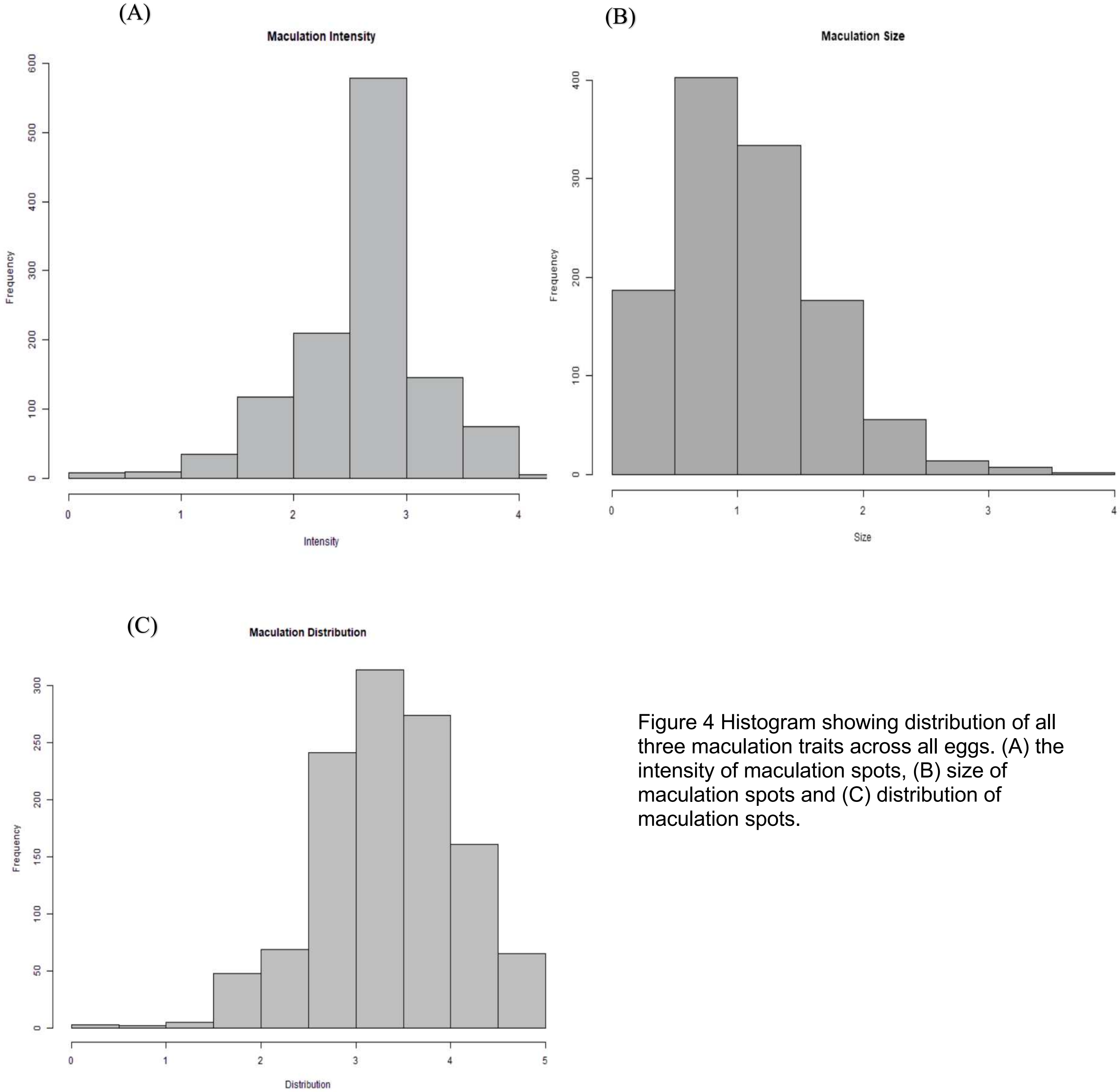

Figure 4 Histogram showing distribution of all three maculation traits across all eggs. (A) the intensity of maculation spots, (B) size of maculation spots and (C) distribution of maculation spots.

Figure 4 shows us the distribution of all three maculation traits (intensity, size and distribution) after scoring, according to our findings maculation intensity (A) shows consistent values across all eggs mainly between (2.5 – 3), which suggested us that very low or high maculation intensity was rare in our data. In case of maculation size (B) the histogram is right-skewed with most of the eggs ranging from (0.5 – 1) showing us that most females laid eggs with relatively small sized maculation spots.

However, in case of distribution (C) we saw slightly right-skewed histogram telling us that most females laid eggs with moderate distribution score between (2.5 – 4). Some females also laid eggs with distribution score of 5.

Variation in egg mass from the year 2008 to 2022 has been illustrated in Figure 5. The value of egg mass fluctuated within a consistent range over 15-year period, although slight year to year variation is also visible.

The median egg mass remained stable mostly centred around 1.15 to 1.20 grams. In years 2014 to 2017 a slight decline is noticeable in both median and interquartile range. The years 2020 and 2021 further show decrease in median egg mass, which suggests a possible downward trend during that period. However, in 2022 there is a rebound between median and upper quartile.

In year 2015 and 2022 outliers were present showing unusually high or low egg mass value other than that outliers were present in most of the years. The overall distribution suggests consistence pattern with no extreme shifts.

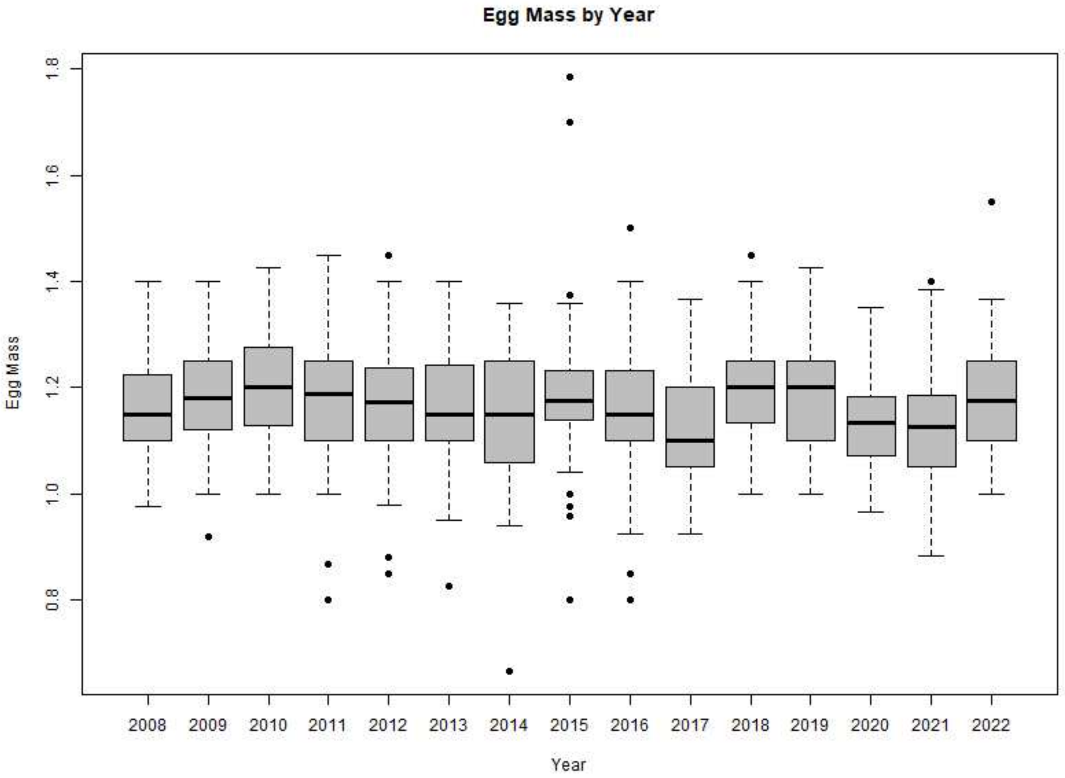

Figure 4 Bar graph showing variation in egg mass from year 2008-2022.The boxplots display the distribution of egg mass data for each year, highlighting the median (central line), interquartile range (box), and potential outliers (circles).

3.1.2 Observations Per Female

A total of 1184 observations were recorded across 796 unique females in dataset. This shows that over the course of the study some females were sampled more than once. Each female was observed approximately 1-2 times as shown in Table 2.

Table 2 Summary of female observations in the dataset, including the mean, minimum and maximum repeat observations per individual, including total unique females and total recorded observations

3.2 With-in Clutch Repeatability: Comparison between Eggs from Same Clutch

In an order to evaluate the similarity of Maculation traits (Intensity, Distribution and Size) between two different eggs from same clutch, we conducted Pearson correlation analyses. According to the results we got there is a strong and statistically significant correlation between all three traits ($p < 2.2 \times 10^{-16}$), that suggests high consistency between appearance of eggs with-in individual clutch.

Among all the maculation traits size showed the highest correlation (r = 0.695,95% Cl: 0.665-0.724), which is then followed by intensity (r = 0.667, 95% Cl: 0.634-0.697) and in the end Distribution had a reasonably strong correlation (r = 0.494, 95% Cl: 0.449-0.536), which indicated that even though all the traits are expressed steadily, Distribution of spots may exhibit more variability with-in clutch.

Table 3 Correlation between maculation traits different eggs within clutch. Pearson correlation coefficient with 95% confidence intervals all p values are highly significant (< 2.2e-16).

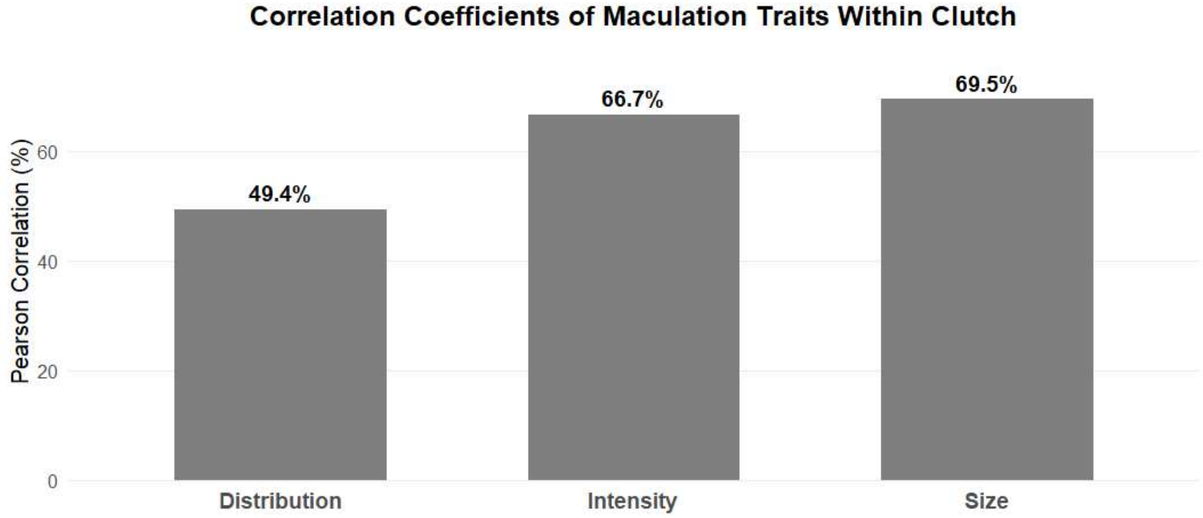

Figure 5 Pearson correlation coefficient of maculation traits within clutch. All the traits here show steady to strong positive correlation, which indicates consistent trait expression between traits.

3.3 Univariate models

Table 4 Variance components, standard errors (SE), Z ratios and associated p values for Egg Mass, significant effects are in bold ($p < 0.05$). Heritability (h^2) = 0.339 (SE = 0.125).

Table 4 and Figure 7 is highlighting the role of genetic factors that are determining egg mass. According to our results additive genetic effects have the largest proportion of variance after residual(unexplained) about 33-34 % with heritability of 0.339 (SE=0.12) that gives us moderate genetic basis for this trait. These results are further supported by the significant value of Z-ratio (Z = 2.70. Nest box and individual effect giving us lowest values of Z-ratio and being non-significant. Meanwhile year (annual variation) suggested minor yet significant variation with Z = 2.00.

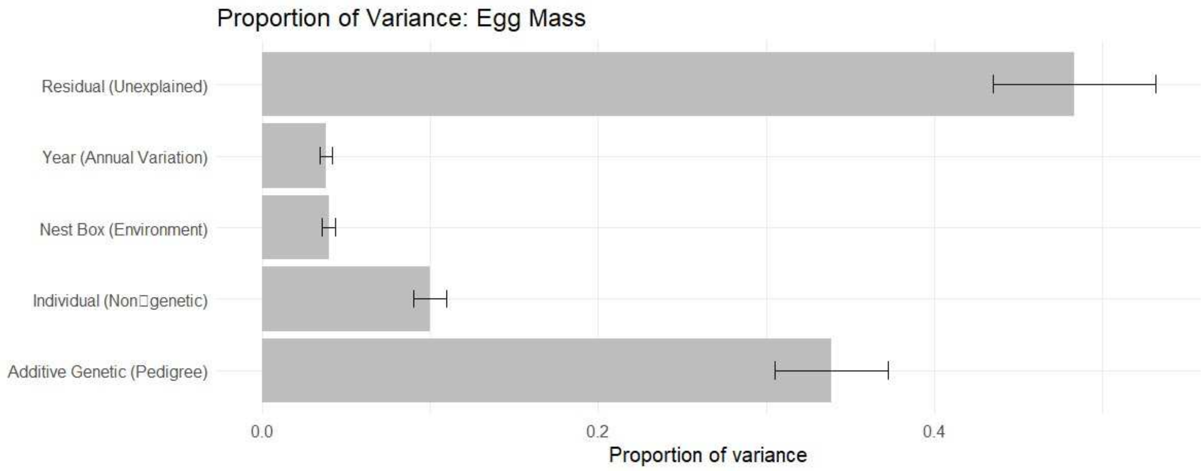

Figure 6 Bar graph showing proportion of variance attributed to additive genetic effect (pedigree), individual (non-genetic), nest box (environmental) and year (annual) variance. Error bars representing standard errors around estimated variance components.

Table 5 Variance components, standard errors (SE), Z ratios and associated p values for Egg Maculation Intensity, significant effects are in bold ($p < 0.05$). Heritability (h^2) = 0.403 (SE = 0.114).

In case of maculation intensity additive genetic component is accounted for 40% of the total variance as shown in Figure 8, these results are further supported in Table 5 where Z-ratio is 3.54 indicating significant genetic control on intensity of maculation. The heritability in case of maculation intensity was estimated at 0.403 (SE= 0.114) which suggests us moderate to high potential for evolutionary response. Furthermore individual (non-genetic) and nest box (environmental) have variance estimate and Z-ratios of zero and are absent from the graph (Figure 8). Meanwhile year effect (annual variation) contributed approximately 15% of the variance with Z-ratio of 2.39.

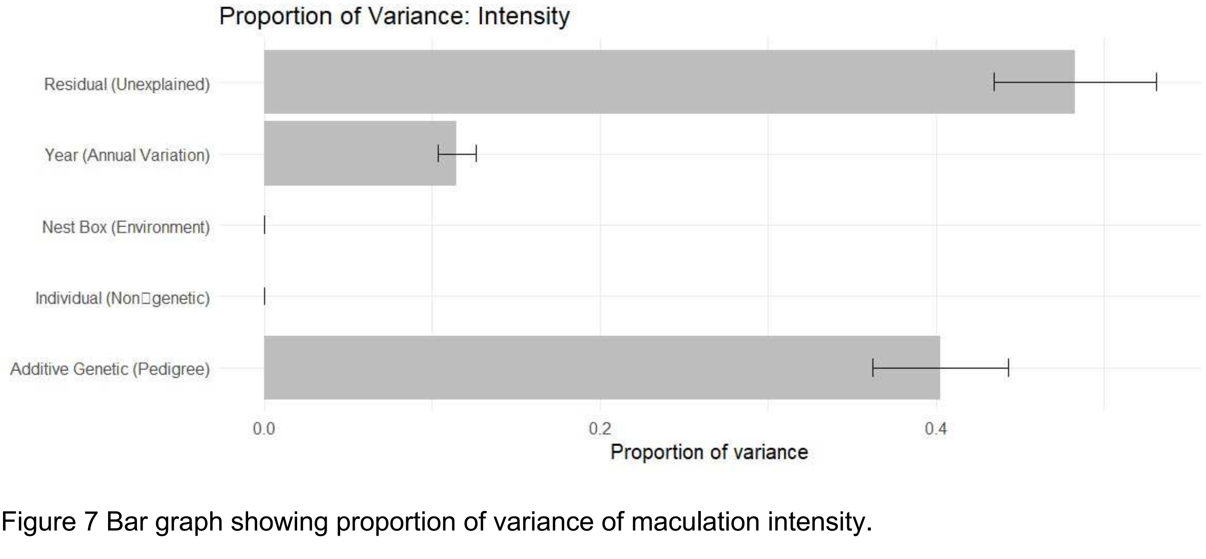

Figure 7 Bar graph showing proportion of variance of maculation intensity.

Table 6 Variance components, standard errors (SE), Z ratios and associated p values for Egg Maculation Distribution, significant effects are in bold ($p < 0.05$). Heritability (h^2) = 0.155 (SE = 0.111).

Table 6 and Figure 9 explains the role of genetic factors that are determining maculation distribution. According to our results additive genetic effects does not have any significant effect with heritability estimate of 0.155 give us the idea that maculation distribution is not linked with genetics. However, random effect of year (annual variation) showed a significant value ($p<0.05$) which indicated that annual variation plays a significant role in distribution of eggshell spots.

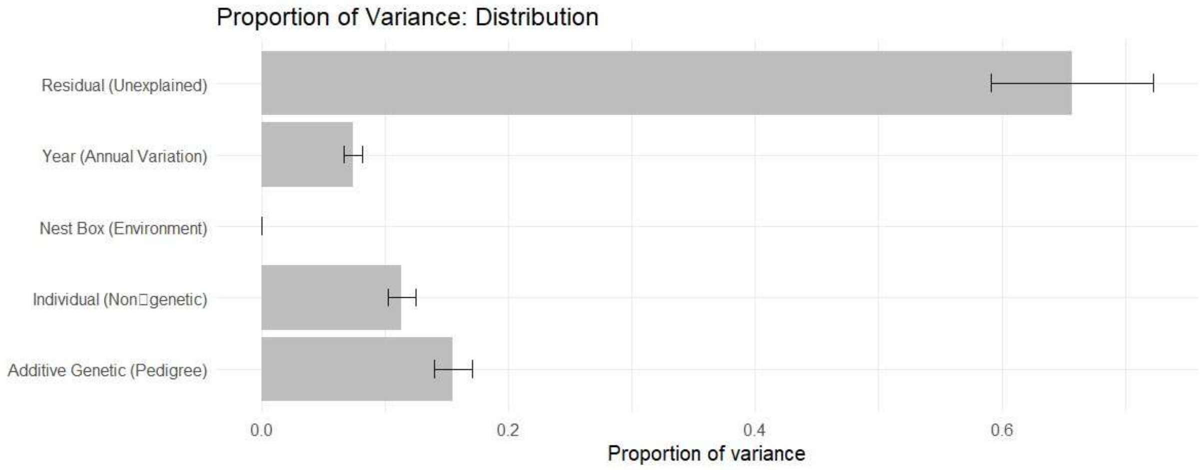

Figure 8 Bar graph presenting proportion of variance for maculation distribution.

Table 7 Variance components, standard errors (SE), Z ratios and associated p values for Egg Maculation Size, significant effects are in bold ($p < 0.05$). Heritability (h^2) = 0.325 (SE = 0.083).

In case of maculation size additive genetic shows, us the significant effect with heritability of 0.325 and (SE= 0.083) (Table 7). However random effect year (annual variation) also showed a significant variance ($p < 0.01$) indicating that the size of maculation spots on eggshells are also affect by it. (Figure 10) represents the proportion of variance of different effects more clearly.

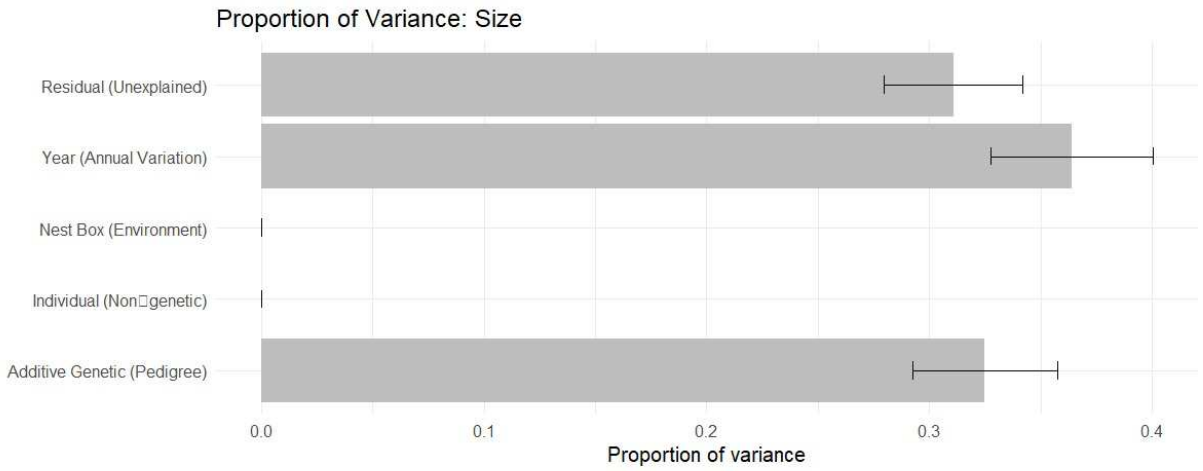

Figure 9 Bar graph showing proportion of variance for maculation size.

3.4 Multivariate Mixed Model and Genetic Correlation

Genetic correlation matric (A) among maculation traits (size, distribution and intensity) is shown in Figure 11. According to our analyses a strong correlation was observed between maculation intensity and size with r = 0.67 which suggested us that eggs with more intense spots have larger spots. On the other hand, distribution with both intensity and size showed a negative correlation r = - 0.37 and r = - 0.36 respectively, giving us idea about how dependent these traits. On the right side we have stacked bar plot representing variance portioning by traits (B) showing us contribution of variance components for each trait together. These patterns tell us that while maculation intensity is highly determined by genetics, distribution and size on the other hand are somewhat more defined by non-genetic and environmental factors.

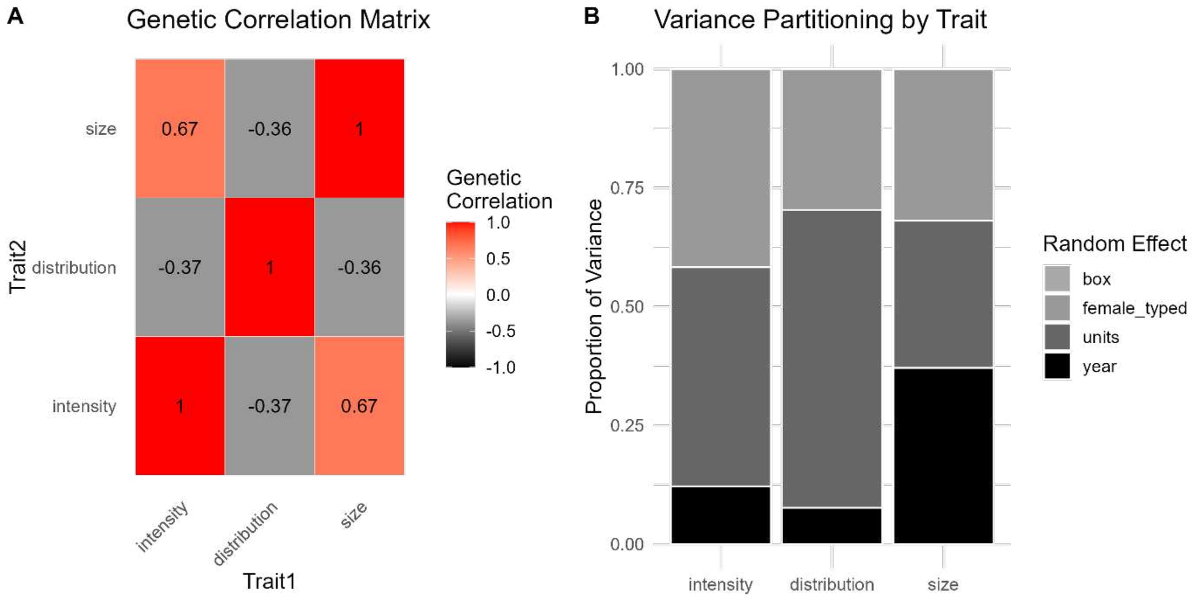

Figure 10 **(A)** shows the heat-map of genetic pair-wise correlations between all the maculation traits that were measured, colour of cells scales from black indicating strong negative to white indicating zero to red which indicated strong positive, values inside each of the cells tells us the posterior-mode correlation estimate. **(B)** Partitioning of phenotypic variance for the same traits, which are expressed as the proportion attributable to each random effect in our multivariate animal model, stacked bars sum to one for every trait, which are highlighting the importance of random effects are different across each trait.

Genetic covariance for each trait within themselves and with other maculation traits were analysed (Table 8) and according to our results distribution of maculation spots showed negative relationship with intensity and size (-0.053 and -0.048 respectively) whereas, intensity and size showed a positive covariance (0.0091) which was also the case in Genetic correlation matrix (Figure 11)

Table 8 Genetic Covariance Matrix for maculation intensity, distribution and size, values that are shown diagonally present variance genetic variance of each trait whereas, values presented off diagonally present genetic covariance between traits.

	Intensity	**Distribution**	**Size**
Intensity	0.150	-0.053	0.091
Distribution	-0.053	0.148	-0.048
Size	0.091	-0.048	0.116

4 Discussion

This thesis primarily investigates the maculation traits (intensity, distribution, and size) of eggshell spottiness in blue tits and quantifies their heritability to ascertain their genetic inheritance. Initially, I extended the research of Cribellier J. (2021) by evaluating the maculation traits through the scoring methodology of Gosler, discovering that blue tits exhibit significant repeatability in the scoring of identical eggs. Subsequently, we discovered that the egg mass remained stable from 2008 to 2022, exhibiting no significant fluctuations. We also noted that eggshell spottiness remains consistent within clutches over time. Our heritability estimates indicated that egg mass, spot size, and spot intensity are heritable traits, whereas the distribution of spots was not significantly affected by genetic factors, aligning with findings from studies on great tits (Gosler et al. 2005). Ultimately, we identified genetic correlations among various maculation traits, indicating that eggs with larger spots generally exhibit more pronounced spots.

4.1 Statistics for maculation traits and egg mass

According to our results among three maculation traits we found that most of the females lay egg with consistent spot intensity that means that blue tits females don’t lay eggs with very high or low intensity score. In case of size of spots, we found out that females laid eggs with relatively small sized eggs, in case of distribution even though we found moderate distribution of spots among most of the female eggs, but some eggs were found with high distribution score.

Furthermore, egg mass was also found to be consistent throughout study period there were no extreme shifts found a slight difference was visible through 2008 to 2022 but nothing significant.

4.2 Within clutch repeatability

After using Gosler et al.'s (2005) scoring method on all maculation traits (intensity, distribution, and size), we conducted Pearson's correlation analyses, and we found that all three traits showed high consistency within a clutch with a robust, significant correlation, which is precisely what we expected. Although distribution of spots may exhibit more variability than maculation size and intensity, it still showed a significant correlation. However, we need to acknowledge several limitations here. First, we only used two eggs per clutch. This small sample may not be able to capture the range of variation within the clutch, thus leading to an overestimation of repeatability. Secondly, the scoring method may sometimes introduce measurement errors. The method we used (Gosler) is based on visual assessment and subjective categorization, which sometimes could lead to inconsistencies between observers or even within the same observer.

In the future, we aim to improve this sampling by increasing the sample size and by using more objective and automated image analysis techniques for scoring maculation traits, which will reduce observer bias and increase the reliability of the data.

4.3 Univariate model and variance partitioning

For this model, we expected all the maculation traits to be heritable, along with egg mass, to give us solid evidence regarding maternal involvement. In this model, we first used the variance partitioning approach. We use this method to find an estimate of how much of the variation in our maculation trait and mass of the egg across years is due to genetic components and how much is due to other factors like environmental components. Here, we included female age and tarsus length as fixed effects in our model, both of which are known to influence egg characteristics (Senesi et al., 2015, Valcu et al. 2024). Our aim behind this was to isolate the genetic signal more accurately and reduce the potential confounding from physiological and age-related variation. What we found was that among all maculation traits, the intensity of spots on eggshell is the most heritable trait with statically significant p value, after that we have size of maculation spots which also showed statically significant value however, in case of Distribution of maculation spots we found that Distribution of spots is more influenced by year (annual variation). From these results, we can suggest that the intensity and size of spots on eggshell have more potential to respond to natural or sexual selection and are more tightly linked with maternal condition, as demonstrated by Cribellier (2021) in previous studies that eggshell spottiness is a trait linked to females. Then we used the same approach for the mass of the egg, and we found that the mass of the egg is also a heritable trait with a statistically significant p-value. Our results are consistent with various previous studies (Kvalnes et al., 2013; Christians, J., 2002).

4.4 Multivariate model

In this model, we are expecting to find a correlation between maculation traits and the extent to which they can evolve independently. In this model, we used an approach implemented by Covarrubias-Pazaran (2016) to find out the genetic correlation between all maculation traits.

We found out that eggs with bigger spots tend to have more intense spots, which indicates that overlapping sets influence these two traits. In evolutionary terms, this means that a change in one trait is highly likely to be accompanied by a correlated response in the other. For example, selection for larger spots may lead to more intense spots, potentially guiding the evolution of the trait.

However, the Distribution of maculation spots did not show any evolutionary relationship with the rest of the traits. Results from these analyses also strengthen the finding from the previous model, which told us that the Distribution of spots is not a heritable trait. However, we could not find the standard errors or perform a Z-test to determine significant variance due to convergence issues. In the future, we tend to alternate modelling approaches to help us solve those issues and get clearer insights.

4.5 Conclusion

Intensity and size of eggshell spottiness in blue tits are a heritable trait and can response to evolution whereas distribution of spots is more linked with environmental factors. Mass of egg in blue tits is also a heritable trait. Eggs with bigger sized spots tends to have more intense spots. Future studies should aim to improve the sample size and scoring techniques, which will give us better understanding of understanding genetic architecture of eggshell maculation.

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References

Ahmad, S. F., Khan, N. N., Ganai, N. A., Shanaz, S., Rather, M. A., & Alam, S. (2021). Multivariate quantitative genetic analysis of body weight traits in Corriedale sheep. Tropical animal health and production, 53(2), 197. https://doi.org/10.1007/s11250-021-02632-3

Attard, M. R. G., Bowen, J., & Portugal, S. J. (2023). Surface texture heterogeneity in maculated bird eggshells. Journal of the Royal Society Interface, 20(20230293). https://doi.org/10.1098/rsif.2023.0293

Bakken, G.S., Vanderbilt, V.C., Buttemer, W.A. & Dawson, W.R. (1978) Avian eggs:

Charmantier, A., Doutrelant, C., Dubuc-Messier, G., Fargevieille, A., & Szulkin, M. (2015). Mediterranean blue tits as a case study of local adaptation. Evolutionary applications, 9(1), 135–152. https://doi.org/10.1111/eva.12282.

Christians J. K. (2002). Avian egg size: variation within species and inflexibility within individuals. Biological reviews of the Cambridge Philosophical Society, 77(1), 1–26. https://doi.org/10.1017/s1464793101005784

Covarrubias-Pazaran G (2016) Genome-Assisted Prediction of Quantitative Traits Using the R Package sommer. PLoS ONE 11(6): e0156744. https://doi.org/10.1371/journal.pone.0156744

Cramp, S. (Ed.). (1998). The complete birds of the Western Palearctic on CD-ROM. Oxford University Press.

Fernandez-Duque, F., Stanback, M., Lawson, S. L., & Hauber, M. E. (2025). Egg shape and color mediate acceptance thresholds in diverse avian host species with different rates of antiparasitic egg rejection. The Journal of experimental biology, 228(3), JEB249396. https://doi.org/10.1242/jeb.249396

Fossøy, F., Sorenson, M. D., Liang, W., Ekrem, T., Moksnes, A., Møller, A. P., Rutila, J., Røskaft, E., Takasu, F., Yang, C., & Stokke, B. G. (2016). Ancient origin and maternal inheritance of blue cuckoo eggs. Nature communications, 7, 10272. https://doi.org/10.1038/ncomms10272

Gosler AG, Barnett PR, Reynolds SJ (2000) Inheritance and variation in eggshell patterning in the

Gosler AG, Higham JP, Reynolds SJ (2005) Why are birds´eggs speckled? Ecol Lett 8:1105–1113.

great tit Parus major. Proc R Soc Lond B 270:2469-2473.

Hancock, G. R. A., & Troscianko, J. (2022). CamoEvo: An open access toolbox for artificial camouflage evolution experiments. Evolution; international journal of organic evolution, 76(5), 870–882. https://doi.org/10.1111/evo.14476

Higham, J. P., & Gosler, A. G. (2006). Speckled eggs: water-loss and incubation behaviour in the great tit Parus major. Oecologia, 149(4), 561-570.

Holveck, M. J., Grégoire, A., Staszewski, V., Guerreiro, R., Perret, P., Boulinier, T., & Doutrelant, C. (2012). Eggshell spottiness reflects maternally transferred antibodies in blue tits. PloS one, 7(11), e50389.

Hugo Bonat W. (2017). Modelling Mixed Types of Outcomes in Additive Genetic Models. The international journal of biostatistics, 13(2), /j/ijb.2017.13.issue-2/ijb-2017-0001/ijb-2017-0001.xml. https://doi.org/10.1515/ijb-2017-0001

Kennedy, G. Y., & Vevers, H. G. (1976). A survey of avian eggshell pigments. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 55(1), 117-123.

Kilner, R. M. (2006). The evolution of egg colour and patterning in birds. Biological Reviews, 81(3), 383-406.

Kim, S. Y., Fargallo, J. A., Vergara, P., & Martínez-Padilla, J. (2013). Multivariate heredity of melanin-based coloration, body mass and immunity. Heredity, 111(2), 139–146. https://doi.org/10.1038/hdy.2013.29

L'Herpiniere, K. L., Tims, A. R., Englert Duursma, D., & Griffith, S. C. (2021). The evolution of egg colour and patterning in Australian songbirds. Evolution, 75(12), 3132-3141.

Lack, D. (1971). Ecological isolation in birds (pp. 228-233). Oxford: Blackwell Scientific Publications.

McCambridge, H. 2024. Blue tit diary. British Trust for Ornithology. https://www.bto.org/understanding-birds/articles/blue-tit-diary

MICHAEL I. CHERRY, ANDREW G. GOSLER, Avian eggshell coloration: new perspectives on adaptive explanations, Biological Journal of the Linnean Society, Volume 100, Issue 4, August 2010, Pages 753–762, https://doi.org/10.1111/j.1095-8312.2010.01457.x

Moreno, J., & Osorno, J. L. (2003). Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? Ecology Letters, 6(9), 803-806.

Naturhistoriska.

Nord, A., & Nilsson, J. Å. (2011). Incubation temperature affects growth and energy metabolism in blue tit nestlings. The American Naturalist, 178(5), 639–651.

Orłowski, G., Niedzielski, P., Merta, D. et al. Quantifying the functional disparity in pigment spot-background egg colour ICP-OES-based eggshell ionome at two extremes of avian embryonic development. Sci Rep 10, 22107 (2020). https://doi.org/10.1038/s41598-020-79040-4

Senesi et al. (2015) – European quail, age and egg quality:

Senesi, E., Ferretti, M., Zappa, A., Luzi, F., & Petrera, F. (2015). Effect of the age of female breeders on egg quality and chick performance in the European quail (Coturnix coturnix). Revista Brasileira de Ciência Avícola, 17(3), 355–360. https://doi.org/10.1590/1516-635x1703355-360

Stenning, M. (2018). The blue tit (p. 17). Bloomsbury Publishing.

Svensson, L. (1984) Identification guide to European passerines, 3rd edn. Stockholm:

Temizyürek, T., Johannknecht, M., & Korsten, P. (2024). Incubation before clutch completion predicts incubation time and hatching asynchrony in the blue tit Cyanistes caeruleus. Ardea, 110(2), 213-231.

Teplitsky, C., Mills, J. A., Yarrall, J. W., & Merilä, J. (2009). Heritability of fitness components in a wild bird population. Evolution, 63(3), 716-726.

thermoregulatory value of very high near-infrared reflectance. Science, 200, 321–323.

Valcu et al. (2024) – Blue tit, female age and egg composition:

Valcu, M., Poláček, M., & Kempenaers, B. (2024). Female age and laying order shape egg composition in a wild bird. *bioRxiv*. https://doi.org/10.1101/2024.05.29.595268

Variation of eggshell spottiness in a wild blue tit (Cyanistes caeruleus) population of southwestern Finland. MSc thesis University of Orléans, France